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09/700806

INTERNATIONAL APPLICATION NO.
PCT/US00/30294INTERNATIONAL FILING DATE
November 2, 2000PRIORITY DATE CLAIMED
November 2, 1999

TITLE OF INVENTION MODULATION OF eNOS ACTIVITY AND THERAPEUTIC USES THEREOF

APPLICANT(S) FOR DO/EO/US Ben-Quan Shen, Thomas Zioncheck

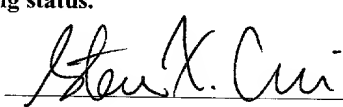
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ has been communicated by the International Bureau.
 - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☒ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A **FIRST** preliminary amendment.
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
Petition for Early Filing Date Under 35 U.S.C. § 102(e)

09/700806

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|---|--------------|--|-------------------|--|--------|
| U.S. APPLICATION NO. 09/7700806 | | INTERNATIONAL APPLICATION NO. PCT/US00/30294 | | ATTORNEY'S DOCKET NUMBER P1735R1 | |
| 17. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) : Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1000.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT = | | | | CALCULATIONS PTO USE ONLY | |
| | | | | | |
| Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)). | | | | \$ | |
| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE | | |
| Total claims | - 20 = | | X \$18.00 | \$ | |
| Independent claims | - 3 = | | X \$80.00 | \$ | |
| MULTIPLE DEPENDENT CLAIM(S) (if applicable) | | | + \$270.00 | \$ | |
| TOTAL OF ABOVE CALCULATIONS = | | | | \$ | 860.00 |
| <input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. | | | | \$ | |
| SUBTOTAL = | | | | \$ | 860.00 |
| Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)). | | | | \$ | |
| TOTAL NATIONAL FEE = | | | | \$ | 860.00 |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property | | | | \$ | |
| TOTAL FEES ENCLOSED = | | | | \$ | 860.00 |
| | | | | Amount to be refunded: | \$ |
| | | | | charged: | \$ |
| a. <input type="checkbox"/> A check in the amount of \$_____ to cover the above fees is enclosed. | | | | | |
| b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. <u>07-0630</u> in the amount of \$ <u>860.00</u> to cover the above fees. A duplicate copy of this sheet is enclosed. | | | | | |
| c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>07-0630</u> . A duplicate copy of this sheet is enclosed. | | | | | |
| NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status. | | | | | |
| SEND ALL CORRESPONDENCE TO. GENENTECH, INC. 1 DNA Way South San Francisco, CA 94080-4990 | | | | | |
| | | | |  SIGNATURE: | |
| | | | | Steven X. Cui NAME | |
| | | | | <u>44,637</u> REGISTRATION NUMBER | |

MODULATION OF eNOS ACTIVITY AND THERAPEUTIC USES THEREOFFIELD OF THE INVENTION

The present invention relates to the use of VEGF, and variants thereof, VEGF receptor agonists, and other agents to modulate the endothelial nitric oxide synthase (eNOS) activity. In particular, modulation of eNOS activity is used to treat or prevent mammalian diseases or disorders associated with vascular endothelial cell dysfunction.

BACKGROUND OF THE INVENTION

The two major cellular components of the vasculature are the endothelial and smooth muscle cells. The endothelial cells form the lining of the inner surface of all blood vessels and constitute a nonthrombogenic interface between blood and tissue. In addition, endothelial cells are an important component for the development of new capillaries and blood vessels. Thus, endothelial cells proliferate during the angiogenesis, or neovascularization, associated with tumor growth and metastasis, as well as a variety of non-neoplastic diseases or disorders.

Various naturally occurring polypeptides reportedly induce the proliferation of endothelial cells. Among those polypeptides are the basic and acidic fibroblast growth factors (FGF), Burgess and Maciag, *Annual Rev. Biochem.*, 58:575 (1989), platelet-derived endothelial cell growth factor (PD-ECGF), Ishikawa *et al.*, *Nature*, 338:557 (1989), and vascular endothelial growth factor (VEGF), Leung *et al.*, *Science*, 246:1306 (1989); Ferrara and Henzel, *Biochem. Biophys. Res. Commun.*, 161:851 (1989); Tischer *et al.*, *Biochem. Biophys. Res. Commun.*, 165:1198 (1989); Ferrara *et al.*, PCT Pat. Pub. No. WO 90/13649 (published November 15, 1990).

VEGF has been reported as a key regulator of angiogenesis and vasculogenesis. Ferrara and Davis-Smyth (1997) *Endocrine Rev.* 18:4-25. Compared to other growth factors that contribute to the processes of vascular formation, VEGF is unique in its high specificity for endothelial cells. It is important not only for normal physiological processes such as wound healing, the female reproductive tract, bone/cartilage formation and embryonic formation, but also during the development of conditions or diseases that involve pathological angiogenesis, for example, tumor growth, age-related macular degeneration (AMD) and diabetic retinopathy. Ferrara and Davis-Smyth (1997), *supra*; Folkman J. (1995) *Nature Med.* 1:27-31; Pepper MS. (1997) *Arterioscler Thromb. Vasc. Biol.* 17:605-619.

In addition to being an angiogenic factor in angiogenesis and vasculogenesis, VEGF, as a pleiotropic growth factor, exhibits multiple biological effects in other physiological and pathological processes, such as endothelial cell survival, vessel permeability and vasodilation, monocyte chemotaxis and calcium influx. Ferrara and Davis-Smyth (1997), *supra*.

VEGF was first identified in media conditioned by bovine pituitary follicular or folliculostellate cells. Biochemical analyses indicate that bovine VEGF is a dimeric protein with an apparent molecular mass of approximately 45,000 Daltons and with an apparent mitogenic specificity for vascular endothelial cells. DNA encoding bovine VEGF was isolated by screening a cDNA library prepared from such cells, using oligonucleotides based on the amino-terminal amino acid sequence of the protein as hybridization probes.

Human VEGF was obtained by first screening a cDNA library prepared from human cells, using bovine VEGF cDNA as a hybridization probe. One cDNA identified thereby encodes a 165-amino acid protein having greater than 95% homology to bovine VEGF; this 165-amino acid protein is typically referred to as human VEGF (hVEGF) or VEGF₁₆₅. The mitogenic activity of human VEGF was confirmed by expressing the human VEGF cDNA in mammalian host cells.

Media conditioned by cells transfected with the human VEGF cDNA promoted the proliferation of capillary endothelial cells, whereas control cells did not. [See Leung et al., *Science*, 246:1306 (1989)].

Although a vascular endothelial cell growth factor could be isolated and purified from natural sources for subsequent therapeutic use, the relatively low concentrations of the protein in follicular cells and the high cost, both in terms of effort and expense, of recovering VEGF proved commercially unavailing. Accordingly, further efforts were undertaken to clone and express VEGF via recombinant DNA techniques. [See, e.g., *Laboratory Investigation*, 72:615 (1995), and the references cited therein].

VEGF is expressed in a variety of tissues as multiple homodimeric forms (121, 145, 165, 189, and 206 amino acids per monomer) resulting from alternative RNA splicing. VEGF₁₂₁ is a soluble mitogen that does not bind heparin; the longer forms of VEGF bind heparin with progressively higher affinity. The heparin-binding forms of VEGF can be cleaved in the carboxy terminus by plasmin to release a diffusible form(s) of VEGF. Amino acid sequencing of the carboxy terminal peptide identified after plasmin cleavage is Arg₁₁₀-Ala₁₁₁. Amino terminal "core" protein, VEGF (1-110) isolated as a homodimer, binds neutralizing monoclonal antibodies (such as the antibodies referred to as 4.6.1 and 3.2E3.1.1) and soluble forms of FLT-1 and KDR receptors with similar affinity compared to the intact VEGF₁₆₅ homodimer.

Certain VEGF-related molecules have also been identified. Ogawa et al. described a gene encoding a polypeptide (called VEGF-E) with about 25% amino acid identity to mammalian VEGF. The VEGF-E was identified in the genome of Orf virus (NZ-7 strain), a parapoxvirus that affects sheep and goats and occasionally, humans, to generate lesions with angiogenesis. The investigators conducted a cell proliferation assay and reported that VEGF-E stimulated the growth of human umbilical vein endothelial cells as well as rat liver sinusoidal endothelial cells to almost the same degree as human VEGF. Binding studies were also reported. A competition experiment was conducted by incubating cells that overexpressed either the KDR receptor or

the FLT-1 receptor with fixed amounts of ¹²⁵I-labeled human VEGF or VEGF-E and then adding increasing amounts of unlabeled human VEGF or VEGF-E. The investigators reported that VEGF-E selectively bound KDR receptor as compared to FLT-1. [Ogawa et al. *J. Biological Chem.* 273:31273-31281 (1998)].

Meyer et al., *EMBO J.*, 18:363-374 (1999), have also identified a member of the VEGF family which is referred to as VEGF-E. The VEGF-E molecule reported by Meyer et al. was identified in the genome of Orf virus strain D1701. *In vitro*, the VEGF-E was found to stimulate release of tissue factor and stimulate proliferation of vascular endothelial cells. In a rabbit *in vivo* model, the VEGF-E stimulated angiogenesis in the rabbit cornea. Analysis of the binding properties of the VEGF-E molecule reported by Meyer et al., in certain assays revealed the molecule selectively bound to the KDR receptor as compared to the FLT-1 receptor.

Olofsson et al., *Proc. Natl. Acad. Sci.*, 95:11709-11714 (1998) report that a protein referred to as "VEGF-B" selectively binds FLT-1. The investigators disclose a mutagenesis experiment wherein the Asp63, Asp64, and Glu67 residues in VEGF-B were mutated to alanine residues. Analysis of the binding properties of the mutated form of VEGF-B revealed that the mutant protein exhibited a reduced affinity to FLT-1.

VEGF contains two sites that are responsible respectively for binding to the KDR (kinase domain region) and FLT-1 (FMS-like tyrosine kinase) receptors. These receptors are believed to exist primarily on endothelial (vascular) cells. Recently, Soker et al. identified another VEGF receptor that has a sequence identical to neuropilin. Soker et al., *Cell*, 92:735-745 (1998). This receptor bound to VEGF₁₆₅ and placental growth factor-2 (PLGF-2) but not to VEGF₁₂₁. Soker et al., *supra*; Migdal et al., *J. Biol. Chem.* 273:22272-22278 (1998).

VEGF production increases in cells that become oxygen-depleted as a result of, for example, trauma and the like, thereby allowing VEGF to bind to the respective receptors to trigger the signaling pathways that give rise to a biological response. For example, the binding of VEGF to such receptors may lead to increased vascular permeability, causing cells to divide and expand to form new vascular pathways - i.e., vasculogenesis and angiogenesis. [See, e.g., Malavaud et al., *Cardiovascular Research*, 36:276-281 (1997)]. It is reported that VEGF-induced signaling through the KDR receptor is responsible for the mitogenic effects of VEGF and possibly, to a large extent, the angiogenic activity of VEGF. [Waltenberger et al., *J. Biol. Chem.*, 269:26988-26995 (1994)]. The biological role(s) of FLT-1, however, is less well understood.

The sites or regions of the VEGF protein involved in receptor binding have been identified and found to be proximately located. [See, Weismann et al., *Cell*, 28:695-704 (1997); Muller et al., *Proc. Natl. Acad. Sci.*, 94:7192-7197 (1997); Muller et al., *Structure*, 5:1325-1338 (1997); Fuh et

al., *J. Biol. Chem.*, 273:11197-11204 (1998)]. The KDR receptor has been found to bind VEGF predominantly through the sites on a loop which contains arginine (Arg or R) at position 82 of VEGF, lysine (Lys or K) at position 84, and histidine (His or H) at position 86. The FLT-1 receptor has been found to bind VEGF predominantly through the sites on a loop which contains aspartic acid (Asp or D) at position 63, glutamic acid (Glu or E) at position 64, and glutamic acid (Glu or E) at position 67. [Keyt et al., *J. Biol. Chem.*, 271:5638-5646 (1996)]. Based on these findings, the wild type VEGF protein has been used as the starting point for introduction of mutations in specific receptor-binding sites, in attempts to create VEGF variants selectively bind to one receptor such as KDR. Keyt et al., *supra*; Shen et al. (1998) *J. Biol. Chem.* 273:29979-29985. The resulting VEGF variants showed moderate receptor selectivity. More recently, based on the crystal structure of VEGF and functional mapping of the KDR binding site of VEGF, it has further been found that VEGF engages KDR receptors using two symmetrical binding sites located at opposite ends of the molecule. Each site is composed of two "hot spots" for binding that consist of residues from both subunits of the VEGF homodimer. Muller et al., *Structure*, 5:1325-1338 (1997). Two of these binding determinants are located within the dominant hot spot on a short, 3-stranded beta-sheet that is conserved in transforming growth factor beta2 (TGF-beta) and platelet-derived growth factor (PDGF).

Recent studies report that endothelium-derived nitric oxide (NO) and endothelial NO synthase (eNOS) may play an important role in various VEGF-induced activities. NO is believed to be an important mediator of endothelial function and a regulator of vascular homeostasis, platelet aggregation, and angiogenesis. Busse and Flemming (1996) *J. Vasc. Res.* 33:181-194. NO is produced from conversion of L-arginine to citrulline by NO-synthase, an enzyme which consists of 3 isoforms denominated endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). Nathan and Xie (1994) *J. Biol. Chem.* 269:13725-13728. VEGF has been shown to induce rapid release of NO from rabbit, pig, bovine and human vascular endothelial cells. (see, e.g., vanderZee et al., *Circulation*, 95:1030-1037 (1997); Parenti et al., *J. Biol. Chem.*, 273:4220-4226 (1998); Morbidelli et al., *Am. J. Physiol.*, 270:H411-H415 (1996); Papapetropoulis et al., *J. Clin. Invest.*, 100:3131-3139 (1997); Kroll et al., *Biochem. Biophys. Res. Comm.*, 252:743-746 (1998); Hood et al., *Am. J. Phys.*, 274:H1054-H1058 (1998)). In an *in vitro* assay, VEGF was found to stimulate human endothelial cells to grow in a NO-dependent manner and promote the NO-dependent formation of vessel-like structures in the 3-D collagen gel model (Papapetropoulis et al., *supra*). Conversely, eNOS inhibitors were reported to inhibit VEGF-induced mitogenic and angiogenic effects. (Papapetropoulis et al., *supra*). In certain *in vivo* studies, inactivation of eNOS expression impaired VEGF-induced angiogenesis in an eNOS knockout mouse model. (Murohara et al., *J.*

Clin. Invest., 101:2567-2578 (1998); Ziche et al., *J. Clin. Invest.*, 99:2625-2634 (1997); Rudic et al., *J. Clin. Invest.*, 101:731-736 (1998)). Recently, Liu et al., *J. Biol. Chem.*, 274:15781-15785 (1999) reported that VEGF down-regulated an endogenous inhibitor of eNOS called Caveolin.

It has been shown that VEGF induces vasodilation in vitro in a dose-dependant fashion and produces transient tachicardia, hypotension and a decrease in cardiac output when injected intravenously in conscious, instrumented rats. Yang et al., *J. Cardiovasc. Pharmacol.*, 27:838-844 (1996) Such acute hemodynamic effects appear to be caused by a decrease in venous return, mediated primarily by endothelial cell-derived NO. Yang et al., *supra*; Hariawala et al., *J. Surg. Res.*, 63:77-82 (1996). Wu et al., *Am. J. Physiol.*, 271:H2735-H2739 (1996) describe that topical application of VEGF resulted in a transient and dose-dependent increase in albumin permeability in isolated coronary venules.

Inhibition of eNOS by L-NAME, *in vivo*, has been reported to result in an increase in mean arterial pressure (MAP) (Yang et.al., 1996 *Cardiovasc. Pharmacol.* 27:838-844; Sase et.al., 1997 *Trends J.Cardiovasc. Med.* 7:28-37) and a decrease in angiogenesis (Papapetropoulos et.al., 1997 *J.Clin. Invest.* 100:3131-3139). Genetically engineered mice lacking the eNOS gene showed impaired endothelium-dependent vasodilation, angiogenesis, and hypertension (Murohara et.al., 1998 *J.Clin. Invest.* 101:2567-2578; Yang et. Al., 1996, *supra*), and over-expression of eNOS in mice by gene transfer was shown to increase nitric oxide production and significantly attenuated MAP and neointima formation (Sase et. al., *supra*; Drummond and Harrison, 1998, *J. Clin. Invest.* 102:2033-2044; Ohashi et.al., 1998 *J. Clin. Invest.* 102:2061-2071).

Although there have been a number of studies on the involvement of VEGF in short term release of NO and eNOS regulation, the chronic effect of VEGF on eNOS expression and/or activity has not been documented. Moreover, biological effects of sustained release of nitric oxide in treating or preventing vascular diseases *in vivo* have not been clearly demonstrated to date. This is due, in large part, to the fact that mammals can rapidly develop tolerance to certain agents exogenously administered as nitric oxide donors, making supplementation of nitric oxide difficult. (Drummond and Harrison, 1998, *J. Clin. Invest.* 102:2033-2044).

Up-regulation of eNOS expression by physiological or pharmacological approaches may provide a useful therapeutic approach to the treatment of diseases associated with endothelial cell dysfunction, for example, by increasing the production and sustained release of endogenous NO.

SUMMARY OF THE INVENTION

The present invention is based on the observation that prolonged VEGF treatment effectively up-regulates eNOS expression and activity, thereby enhancing sustained nitric oxide production, and that the eNOS upregulation

by VEGF requires the VEGF-KDR receptor, activation of the KDR-associated tyrosine kinase (TK) and a downstream PKC-dependent pathway. Modulation of eNOS expression/activity is clinically useful, for example, in the treatment of disorders characterized by endothelial cell dysfunction including eNOS dysfunction and/or defects in nitric oxide production.

Therefore, in one embodiment, the invention provides as claimed a method of treating disorders in mammals wherein nitric oxide is an important regulator, such as hypertension, diabetes, atherosclerosis, thrombosis, angina and heart failure, by modulating eNOS expression or activity, for example, by the administration of an effective amount of VEGF, a receptor-selective VEGF variant, or an agent or molecule which acts as an agonist of VEGF receptor activation.

In another embodiment, the invention provides a method for protecting a mammal from conditions associated with endothelium dysfunction by providing VEGF or VEGF receptor agonist.

In yet another embodiment, the invention provides a method of stimulating a sustained production of endogenous NO in an endothelial cell by providing a receptor selective VEGF variant. Preferably, a KDR selective VEGF variant is used for specifically binding the KDR receptor, which in turn activates the pathway leading to the upregulation of eNOS and sustained NO production.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A-1D depict the eNOS upregulation activity of VEGF. Figure 1A is a representative Western blot showing inducement of a time-dependent increase in eNOS expression by VEGF treatment. The arrow indicates eNOS. Figure 1B is a bar diagram showing densitometry analysis of eNOS levels (means \pm SD, n=3). Figure 1C is a bar diagram illustrating the chronic effect of VEGF on eNOS activity, as expressed by the activity ratio of VEGF-treated cells (after 2-day VEGF exposure) over untreated controls (at Day 0) (means \pm SD, n=3). Figure 1D is a bar diagram illustrating the acute effect of VEGF on eNOS activity, as expressed by the percentage of activity increases of the VEGF-treated cells (treated for 0 to 60 min) over the untreated control.

Figure 2 is a graph showing the fold increase in eNOS protein following treatment with VEGF, L-NAME, SNAP (alone) or a combination of VEGF with L-NAME or SNAP for 0-5 days.

Figures 3A-3C depict the VEGF receptor specificities for eNOS regulation. Figure 3A is a bar diagram showing that VEGF₁₆₅, VEGF₁₁₀, and a KDR selective binding variant ("KDR-sel") induce eNOS up-regulation, whereas FLT-1 receptor selective variants ("FLT-1-sel" and PLGF) did not. Figure 3B is a Western blot showing VEGF-induced eNOS up-regulation in KDR-transfected PAE cells but not in FLT-1 containing PAE cells. Figure 3C is a Western blot showing dose-dependent prevention of VEGF-induced eNOS up-regulation by KDR tyrosine kinase selective inhibitor SU1498. Arrows indicate eNOS.

Figures 4A-4B depict the inhibition of VEGF modulation on eNOS. Figure 4A is a Western blot showing the effects on eNOS expression in ACE cells treated with VEGF in combination with various specific inhibitors of tyrosine kinase, PLC-gamma or PKC. Figure 4B is a bar diagram illustrating analysis of eNOS levels by densitometry.

Figures 5A-5C depict PKC activity in eNOS modulation by VEGF. Figure 5A is a Western blot showing that VEGF treatment resulted in a rapid redistribution of PKC- alpha, gamma and epsilon from cytosolic to membrane fractions. Figure 5B shows that VEGF increased PKC activity. Figure 5C shows that activation of PKC with PMA increased eNOS levels.

Figure 6 is a bar diagram illustrating effects of various angiogenic factors on eNOS expression. The histograms show the fold increase in eNOS protein in growth factor treated cells normalized to untreated control cells.

Figure 7 depicts the ELISA assay titration curve for the native VEGF (8-109).

Figure 8 depicts the KIRA assay titration curve for the native VEGF (8-109).

Figure 9 depicts the HUVEC proliferation assay titration curve for the native VEGF (8-109).

Figure 10 is a Western blot showing the eNOS expression levels in endothelial cells treated by VEGF₁₆₅, VEGF₁₁₀, two KDR selective VEGF variants (KDR-full and KDR-short), or a Flt selective variant (Flt-short).

Figure 11 is a Western blot data showing the in vivo eNOS expression affected by a VEGF antagonist (MuFlt-IgG).

Figure 12 is a Western blot showing the phosphotyrosine levels of eNOS in endothelial cells treated with VEGF for different time courses.

DETAILED DESCRIPTION OF THE INVENTION

A. Definitions

The terms "VEGF" and "native VEGF" as used herein refer to the 165-amino acid vascular endothelial cell growth factor and related 121-, 189-, and 206- amino acid vascular endothelial cell growth factors, as described by Leung *et al.*, *Science*, 246:1306 (1989), and Houck *et al.*, *Mol. Endocrin.*, 5:1806 (1991), together with the naturally occurring allelic and processed forms thereof. The terms "VEGF" and "native VEGF" are also used to refer to truncated forms of the polypeptide comprising amino acids 8 to 109 or 1 to 109 of the 165-amino acid human vascular endothelial cell growth factor. Reference to any such forms of VEGF may be identified in the present application, e.g., by "VEGF (8-109)," "VEGF (1-109)" or "VEGF₁₆₅." The amino acid positions for a "truncated" native VEGF are numbered as indicated in the native VEGF sequence. For example, amino acid position 17 (methionine) in truncated native VEGF is also position 17 (methionine) in native VEGF. The truncated native VEGF has binding affinity for the KDR and FLT-1 receptors comparable to native VEGF.

The term "VEGF variant" as used herein refers to a VEGF polypeptide which includes one or more amino acid mutations in the native VEGF sequence and preferably, has selective binding affinity for either the KDR receptor or the FLT-1 receptor. In one embodiment, the VEGF variant includes one or more amino acid mutations in any one of positions 17 to 25 and/or 63 to 66 of the native VEGF sequence. Optionally, the one or more amino acid mutations include amino acid substitution(s). Optionally, VEGF variants include one or more amino acid mutations and exhibit binding affinity to the KDR receptor which is equal or greater than the binding affinity to the KDR receptor by native VEGF, and preferably, exhibit less binding affinity to the FLT-1 receptor than the binding affinity of native VEGF for FLT-1. When binding affinity of the VEGF variant for the KDR receptor is approximately equal (unchanged) or greater than (increased) as compared to native VEGF, and the binding affinity of the VEGF variant for the FLT-1 receptor is less than or nearly eliminated (as compared to native VEGF), the binding affinity of the VEGF variant is "selective" for the KDR receptor. Alternatively, the VEGF variants include one or more amino acid mutations and exhibit binding affinity to the FLT-1 receptor which is equal or greater than the binding affinity to the FLT-1 receptor by native VEGF, and preferably, exhibit less binding affinity to the KDR receptor than the binding affinity of native VEGF for KDR. When binding affinity of the VEGF variant for the FLT-1 receptor is approximately equal (unchanged) or greater than (increased) as compared to native VEGF, and the binding affinity of the VEGF variant for the KDR receptor is less than or nearly eliminated (as compared to native VEGF), the binding affinity of the VEGF variant is "selective" for the FLT-1 receptor. Preferred VEGF variants of the invention will have at least 10-fold less binding affinity to FLT-1 receptor (as compared to native VEGF), and even more preferably, will have at least 100-fold less binding affinity

Abstract

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Oncogene, 6:1677 (1991); Terman et al., *Biochem. Biophys. Res. Commun.*, 187:1579 (1992).

A receptor "agonist" is an agent that has affinity to and activate a receptor normally activated by a naturally occurring ligand, thus triggering a biochemical response. Generally, the receptor activation capability of the agonist will be at least qualitatively similar (and may be essentially quantitatively similar) to a native ligand of the receptor. Non-limiting examples of a receptor agonist include ligand variant, antibody against the receptor and antibody against the receptor-ligand complex.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts.

Monoclonal antibodies are highly specific, being directed against a single antigen. Furthermore, in contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein.

This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented.

A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the antibody may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antibody to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the antibody are outweighed by the

therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount will be less than the therapeutically effective amount.

"Chronic" administration refers to administration of the agent(s) in a continuous mode as opposed to an acute mode, so as to maintain the initial therapeutic effect (activity) for an extended period of time. Intermittent administration is treatment that is not consecutively done without interruption, but rather is cyclic in nature.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, cats, cows, horses, sheep, pigs, etc.

Preferably, the mammal is human.

Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

Pathologic conditions and disorders "associated with" NO or "characterized by" eNOS dysfunction and/or wherein NO is an "important regulator" are those conditions and disorders where nitric oxide insufficiency or excess is correlated with disease. Such disorders and conditions include, for example, hypertension, diabetes, thrombosis, angina, atherosclerosis, and heart failure, wherein nitric oxide levels in mammalian cells or tissues are present in insufficient quantities as compared to normal or healthy mammalian cells or tissues. Further applications in which the use of VEGF will be beneficial include the use of VEGF to increase eNOS or NO production prior to, concurrent with or subsequent to angioplasty to prevent restenosis or neointima formation.

The terms "hypertension" or "hypertensive condition" as used herein refer to a physiological state or syndrome in mammals typically characterized by increased peripheral vascular resistance or cardiac output, or both. Clinically, "hypertension" or "hypertensive condition" may optionally be indicated by blood pressure measurements equal to or greater than approximately 140 mm Hg systolic and approximately 90 mm Hg diastolic. Hypertension is further characterized in Oates, "Antihypertensive Agents and the Drug Therapy of Hypertension", Chapter 33, Goodman and Gilman, 9th Edition, pages 780-781. The terms as used herein include acute and chronic hypertensive conditions.

As used herein, "modulation" of eNOS activity is used in a broad sense and refers to the ability to induce or enhance, inhibit or decrease, or maintain eNOS protein expression and/or activity.

B. Mode(s) for Carrying out the Invention

In one aspect, the invention provides methods for treating a NO associated disorder or condition such as hypertension, diabetes, thrombosis, angina, heart failure or atherosclerosis. Applicants have observed that some coronary artery disease patients (humans) treated with rhVEGF (either by intracoronary or intravenous infusion administration at 0.05 microgram/Kg body weight/minute) can have a dose-rate dependent reduction in mean arterial pressure. This type of reduction in mean arterial pressure was acute and typically was observed during the first 20 minutes of infusion. In contrast, some cancer patients (humans) treated with recombinant humanized monoclonal antibodies against VEGF can have a dose-dependent increase in mean arterial pressure. Such increase in mean arterial pressure is believed to be due to the neutralization of endogenous VEGF. It is further believed that the blocking or neutralization of VEGF may down-regulate eNOS production or decrease endothelial cell function, affecting the vascular homeostasis and resulting in an increase in blood pressure. Accordingly, VEGF, or molecules modulating VEGF receptor activation, as described herein may be employed to treat conditions or disorders associated with NO or eNOS dysfunction.

The invention also provides methods for protecting a mammalian subject from conditions associated with endothelium dysfunction such as thrombosis.

Early results from a VIVA clinical trial (VEGF in Ischemia for Vascular Angiogenesis) indicate that VEGF treated angina patients show trends of improvement compared to placebo group, in such measures as angina class, angina frequency, and treadmill times at a prolonged time point. It is contemplated that the VEGF or VEGF receptor agonist of the invention provides important vascular protective effects through up-regulating eNOS and NO in endothelial cells. The prolonged treatment of target subject with VEGF or VEGF receptor agonist according to the invention provides chronic effects on eNOS upregulation and sustained production of NO, which are more beneficial for therapeutic treatments or prophylactic measures wherein a sustained level of NO is desired. By boosting the endogenous NO production via upregulating the eNOS enzyme, the methods of the invention are also applicable in treatment or prevention wherein the patients currently rely on exogenous nitrate sources, such as angina patients. Luscher TF (1992) *Br. J. Clin. Pharmacol.* 34 Suppl. 1:29S-35S.

The invention further provides methods of using a KDR selective VEGF variant for stimulating a sustained production of endogenous NO in an endothelial cell. Native VEGF is a pleiotropic growth factor having multiple biological effects in regulating physiological and pathological vascular functions. When used in vitro or in vivo, native VEGF may cause unwanted adverse effects in addition to the targeted function(s), a problem often complicating the therapeutic applications of VEGF. Accordingly, the invention provides methods of using the receptor-selective variants as alternative therapeutic agents that may have fewer side effects than the native VEGF protein.

In one aspect, the VEGF or VEGF receptor agonist of the invention is capable of upregulating the expression level of eNOS in an endothelial cell.

Without being bound to particular mechanisms, the eNOS expression level can be upregulated by increasing the transcription of the eNOS gene, or
5 alternatively, by preventing the degradation of the transcribed eNOS mRNAs, or the combination of both. In another aspect, the VEGF or VEGF receptor agonist of the invention is capable of upregulating the activity of endogenous eNOS. It has been shown that a number of proteins are associated with eNOS and regulate the eNOS activity via protein-protein interactions.
10 For example, it has been shown that caveolin and PLC- γ decrease the eNOS activity, whereas HSP-70 and HSP-90 increase the eNOS activity, when associated with eNOS. Applicants have observed that in cultured endothelial cells, VEGF treatment induces dissociation of caveolin and PLC- γ from eNOS and increase association of eNOS to HSP-70 and HSP-90. Furthermore,
15 prolonged VEGF treatment is shown to reduce the phosphotyrosine level of eNOS, another indication of eNOS activation (Example 14).

VEGF and VEGF variants for use in the disclosed methods may be prepared by a variety of methods well known in the art. Preferably, the VEGF employed in the methods of the present invention comprises recombinant
20 VEGF₁₆₅. Amino acid sequence variants of VEGF can be prepared by mutations in the VEGF DNA. Such variants include, for example, deletions from, insertions into or substitutions of residues within the amino acid sequence shown in Leung *et al.*, *supra* and Houck *et al.*, *supra*. Any combination of deletion, insertion, and substitution may be made to arrive at the final
25 construct having the desired activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure [see EP 75,444A].

The VEGF variants optionally are prepared by site-directed mutagenesis
30 of nucleotides in the DNA encoding the native VEGF or phage display techniques, thereby producing DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture.

While the site for introducing an amino acid sequence variation is predetermined, the mutation *per se* need not be predetermined. For example,
35 to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed VEGF variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well-known, such as, for example, site-specific
40 mutagenesis.

Preparation of the VEGF variants described herein is preferably achieved by phage display techniques, such as those described in the Examples.

After such a clone is selected, the mutated protein region may be removed and placed in an appropriate vector for protein production, generally an expression vector of the type that may be employed for transformation of an appropriate host.

5 Amino acid sequence deletions generally range from about 1 to 30 residues, more preferably 1 to 10 residues, and typically are contiguous.

Amino acid sequence insertions include amino- and/or carboxyl-terminal fusions of from one residue to polypeptides of essentially unrestricted length as well as intrasequence insertions of single or multiple amino acid
10 residues. Intrasequence insertions (i.e., insertions within the native VEGF sequence) may range generally from about 1 to 10 residues, more preferably 1 to 5. An example of a terminal insertion includes a fusion of a signal sequence, whether heterologous or homologous to the host cell, to the N-terminus to facilitate the secretion from recombinant hosts.

15 Additional VEGF variants are those in which at least one amino acid residue in the native VEGF has been removed and a different residue inserted in its place. Such substitutions may be made in accordance with those shown in Table 1.

Table 1

| 20 | Original Residue | Exemplary Substitutions |
|----|------------------|-------------------------|
| | Ala (A) | gly; ser |
| | Arg (R) | lys |
| | Asn (N) | gln; his |
| | Asp (D) | glu |
| 25 | Cys (C) | ser |
| | Gln (Q) | asn |
| | Glu (E) | asp |
| | Gly (G) | ala; pro |
| | His (H) | asn; gln |
| 30 | Ile (I) | leu; val |
| | Leu (L) | ile; val |
| | Lys (K) | arg; gln; glu |
| | Met (M) | leu; tyr; ile |
| | Phe (F) | met; leu; tyr |
| 35 | Ser (S) | thr |
| | Thr (T) | ser |
| | Trp (W) | tyr |
| | Tyr (Y) | trp; phe |
| | Val (V) | ile; leu |

40

Changes in function or immunological identity may be made by selecting substitutions that are less conservative than those in Table 1, i.e., selecting residues that differ more significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the
45 substitution, for example, as a sheet or helical conformation, (b) the

charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. The substitutions that in general are expected to produce the greatest changes in the VEGF variant properties will be those in which (a) glycine and/or proline (P) is substituted by another amino acid or is deleted or inserted; (b) a hydrophilic residue, e.g., seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g., leucyl, isoleucyl, phenylalanyl, valyl, or alanyl; (c) a cysteine residue is substituted for (or by) any other residue; (d) a residue having an electropositive side chain, e.g., lysyl, arginyl, or histidyl, is substituted for (or by) a residue having an electronegative charge, e.g., glutamyl or aspartyl; (e) a residue having an electronegative side chain is substituted for (or by) a residue having an electropositive charge; or (f) a residue having a bulky side chain, e.g., phenylalanine, is substituted for (or by) one not having such a side chain, e.g., glycine.

The effect of the substitution, deletion, or insertion may be evaluated readily by one skilled in the art using routine screening assays.

For example, a phage display-selected VEGF variant may be expressed in recombinant cell culture, and, optionally, purified from the cell culture. The VEGF variant may then be evaluated for KDR or FLT-1 receptor binding affinity and other biological activities, such as those disclosed in the present application. The binding properties or activities of the cell lysate or purified VEGF variant can be screened in a suitable screening assay for a desirable characteristic. For example, a change in the immunological character of the VEGF variant as compared to native VEGF, such as affinity for a given antibody, may be desirable. Such a change may be measured by a competitive-type immunoassay, which can be conducted in accordance with techniques known in the art. The respective receptor binding affinity of the VEGF variant may be determined by ELISA, RIA, and/or BIAcore assays, known in the art and described further in the Examples below. Preferred VEGF variants of the invention will also exhibit activity in KIRA assays (such as described in the Examples) reflective of the capability to induce phosphorylation of the KDR receptor. Preferred VEGF variants of the invention will additionally or alternatively induce endothelial cell proliferation (which can be determined by known art methods such as the HUVEC proliferation assay in the Examples). In addition to the specific VEGF variants disclosed herein, the VEGF variants described in Keyt et al., J. Biol. Chem., 271:5638-5646 (1996) are also contemplated for use in the present invention.

VEGF variants may be prepared by techniques known in the art, for example, recombinant methods. Isolated DNA used in these methods is understood herein to mean chemically synthesized DNA, cDNA, chromosomal, or extrachromosomal DNA with or without the 3'- and/or 5'-flanking regions. Preferably, the VEGF variants herein are made by synthesis in recombinant cell culture.

For such synthesis, it is first necessary to secure nucleic acid that encodes a VEGF or VEGF variant. DNA encoding a VEGF molecule may be obtained from bovine pituitary follicular cells by (a) preparing a cDNA library from these cells, (b) conducting hybridization analysis with labeled DNA encoding the VEGF or fragments thereof (up to or more than 100 base pairs in length) to detect clones in the library containing homologous sequences, and (c) analyzing the clones by restriction enzyme analysis and nucleic acid sequencing to identify full-length clones. If full-length clones are not present in a cDNA library, then appropriate fragments may be recovered from the various clones using the nucleic acid sequence information disclosed herein for the first time and ligated at restriction sites common to the clones to assemble a full-length clone encoding the VEGF. Alternatively, genomic libraries will provide the desired DNA.

Once this DNA has been identified and isolated from the library, it is ligated into a replicable vector for further cloning or for expression.

In one example of a recombinant expression system, a VEGF-encoding gene is expressed in a cell system by transformation with an expression vector comprising DNA encoding the VEGF. It is preferable to transform host cells capable of accomplishing such processing so as to obtain the VEGF in the culture medium or periplasm of the host cell, i.e., obtain a secreted molecule.

"Transfection" refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO_4 and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

"Transformation" refers to introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described by Cohen, *Proc. Natl. Acad. Sci. (USA)*, 69: 2110 (1972) and Mandel et al., *J. Mol. Biol.*, 53: 154 (1970), is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, *Virology*, 52: 456-457 (1978), is preferred. General aspects of mammalian cell host system transformations have been described by Axel in U.S. Pat. No. 4,399,216 issued August 16, 1983. Transformations into yeast are typically carried out according to the method of Van Solingen et al., *J. Bact.*, 130: 946 (1977) and Hsiao et al., *Proc. Natl. Acad. Sci. (USA)*, 76: 3829 (1979). However, other methods for introducing DNA into cells such as by nuclear injection or by protoplast fusion may also be used.

The vectors and methods disclosed herein are suitable for use in host cells over a wide range of prokaryotic and eukaryotic organisms.

In general, of course, prokaryotes are preferred for the initial cloning of DNA sequences and construction of the vectors useful in the invention. For example, *E. coli* K12 strain MM 294 (ATCC No. 31,446) is particularly useful. Other microbial strains that may be used include *E. coli* strains such as *E. coli* B and *E. coli* X1776 (ATCC No. 31,537). These examples are, of course, intended to be illustrative rather than limiting.

Prokaryotes may also be used for expression. The aforementioned strains, as well as *E. coli* strains W3110 (F-, lambda-, prototrophic, ATCC No. 27,325), K5772 (ATCC No. 53,635), and SR101, bacilli such as *Bacillus subtilis*, and other enterobacteriaceae such as *Salmonella typhimurium* or *Serratia marcesans*, and various *pseudomonas* species, may be used.

In general, plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell are used in connection with these hosts. The vector ordinarily carries a replication site as well as marking sequences that are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, e.g., Bolivar et al., *Gene*, 2:95 (1977)). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR322 plasmid, or other microbial plasmid or phage, must also contain, or be modified to contain, promoters that can be used by the microbial organism for expression of its own proteins.

Those promoters most commonly used in recombinant DNA construction include the β -lactamase (penicillinase) and lactose promoter systems [Chang et al., *Nature*, 375:615 (1978); Itakura et al., *Science*, 198:1056 (1977); Goeddel et al., *Nature*, 281:544 (1979)] and a tryptophan (*trp*) promoter system [Goeddel et al., *Nucleic Acids Res.*, 8:4057 (1980); EPO Appl. Publ. No. 0036,776]. While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors [see, e.g., Siebenlist et al., *Cell*, 20:269 (1980)].

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures, may also be used. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example [Stinchcomb et al., *Nature*, 282:39 (1979); Kingsman et al., *Gene*, 7:141 (1979); Tschemper et al., *Gene*, 10:157 (1980)], is commonly used. This plasmid already contains the *trp1* gene that provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44,076 or PEP4-1 [Jones, *Genetics*, 85:12 (1977)]. The presence of the *trp1* lesion as a characteristic of the yeast host cell genome then provides an effective

environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., *J. Biol. Chem.*, 255:2073 (1980)] or other glycolytic enzymes [Hess et al., *J. Adv. Enzyme Reg.*, 7:149 (1968); Holland et al., *Biochemistry*, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions, are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any plasmid vector containing yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years [*Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973)]. Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7, 293, and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus2, and most frequently Simian Virus 40 (SV40). The early and late promoters of SV40 virus are particularly useful because both are obtained easily from the virus as a fragment that also contains the SV40 viral origin of replication [Fiers et al., *Nature*, 273:113 (1978)]. Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250-bp sequence extending from the HindIII site toward the BglI site located in the viral origin of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene

sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) source, or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

Satisfactory amounts of protein are produced by cell cultures; however, refinements, using a secondary coding sequence, serve to enhance production levels even further. One secondary coding sequence comprises dihydrofolate reductase (DHFR) that is affected by an externally controlled parameter, such as methotrexate (MTX), thus permitting control of expression by control of the methotrexate concentration.

In selecting a preferred host cell for transfection by the vectors of the invention that comprise DNA sequences encoding both VEGF and DHFR protein, it is appropriate to select the host according to the type of DHFR protein employed. If wild-type DHFR protein is employed, it is preferable to select a host cell that is deficient in DHFR, thus permitting the use of the DHFR coding sequence as a marker for successful transfection in selective medium that lacks hypoxanthine, glycine, and thymidine. An appropriate host cell in this case is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Natl. Acad. Sci. (USA)*, 77:4216 (1980).

On the other hand, if DHFR protein with low binding affinity for MTX is used as the controlling sequence, it is not necessary to use DHFR-deficient cells. Because the mutant DHFR is resistant to methotrexate, MTX-containing media can be used as a means of selection provided that the host cells are themselves methotrexate sensitive. Most eukaryotic cells that are capable of absorbing MTX appear to be methotrexate sensitive. One such useful cell line is a CHO line, CHO-K1 (ATCC No. CCL 61).

Construction of suitable vectors containing the desired coding and control sequences employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and religated in the form desired to prepare the plasmids required.

If blunt ends are required, the preparation may be treated for 15 minutes at 15°C with 10 units of Polymerase I (Klenow), phenol-chloroform extracted, and ethanol precipitated.

Size separation of the cleaved fragments may be performed using, by way of example, 6 percent polyacrylamide gel described by Goeddel et al., *Nucleic Acids Res.*, 8:4057 (1980).

To confirm correct sequences were constructed in plasmids, the ligation mixtures are typically used to transform *E. coli* K12 strain 294 (ATCC 31,446) or other suitable *E. coli* strains, and successful transformants selected by ampicillin or tetracycline resistance where

appropriate. Plasmids from the transformants are prepared and analyzed by restriction mapping and/or DNA sequencing by the method of Messing et al., *Nucleic Acids Res.*, 9:309 (1981) or by the method of Maxam et al., *Methods of Enzymology*, 65:499 (1980).

5 After introduction of the DNA into the mammalian cell host and selection in medium for stable transfectants, amplification of DHFR-protein-coding sequences is effected by growing host cell cultures in the presence of approximately 20,000-500,000 nM concentrations of methotrexate (MTX), a competitive inhibitor of DHFR activity. The effective range of
10 concentration is highly dependent, of course, upon the nature of the DHFR gene and the characteristics of the host. Clearly, generally defined upper and lower limits cannot be ascertained. Suitable concentrations of other folic acid analogs or other compounds that inhibit DHFR could also be used. MTX itself is, however, convenient, readily available, and effective.

15 Antibodies against the KDR receptor or FLT-1 receptor may also be employed in the methods of the present invention. Optionally, the KDR receptor or FLT-1 receptor antibody is a monoclonal antibody. Optionally, the KDR receptor antibody is an agonist antibody which, preferably, is capable of up-regulating eNOS levels and/or activity. In a hybridoma method
20 for preparing such monoclonal antibodies, a mouse or other appropriate host animal is immunized with antigen by subcutaneous, intraperitoneal, or intramuscular routes to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein(s) used for immunization. Alternatively, lymphocytes may be immunized in vitro.
25 Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell. Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986).

The antigen may be KDR receptor or FLT-1 receptor or optionally, a fragment or portion or epitope or variant thereof having one or more amino
30 acid residues that participate in the binding of hVEGF to its receptors. For example, immunization with an extracellular domain sequence of KDR may especially be useful in producing antibodies that are agonists or antagonists of hVEGF, since it is region(s) within or spanning the extracellular domain that are involved in hVEGF binding. The use of
35 chimeric, anti-idiotypic, humanized or human antibodies against KDR or FLT-1 are contemplated for use in the present invention and may be prepared using techniques known to the skilled artisan.

The VEGF receptor monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No.
40 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once
45 isolated, the DNA may be placed into expression vectors, which are then

transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

The VEGF receptor antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art.

The VEGF receptor antibodies may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art.

Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-327 (1988); Verhoeven et al., *Science*, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies. Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, p. 77 (1985) and Boerner et al., *J. Immunol.*, 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introducing of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Patent Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks et al., *Bio/Technology* 10, 779-783 (1992); Lonberg et al., *Nature* 368 856-859 (1994); Morrison, *Nature* 368, 812-13 (1994); Fishwild et al., *Nature Biotechnology* 14, 845-51 (1996); Neuberger, *Nature Biotechnology* 14, 826 (1996); Lonberg and Huszar, *Intern. Rev. Immunol.* 13 65-93 (1995).

In one embodiment, the therapeutic methods of the present invention include administering VEGF to a mammal to treat hypertension. Hypertension is a relatively common cardiovascular disease in mammals. For instance, elevated arterial pressures can cause pathological changes in the vasculature or hypertrophy of the left ventricle of the heart. Hypertension can result in stroke in some mammals, as well as lead to disease of the coronary arteries or myocardial infarction.

The VEGF of the invention may be formulated and dosed in a fashion consistent with good medical practice taking into account the specific hypertensive condition to be treated, the condition of the individual

patient, the site of delivery of the agent, the method of administration, and other factors known to practitioners. "An effective amount" of VEGF includes amounts that prevent, lessen the worsening of, alleviate, or cure the condition being treated or symptoms thereof. Optionally, "an effective amount" of VEGF is that amount which enhances or up-regulates nitric oxide production in the mammal. Optionally, the VEGF may be used to treat acute conditions of hypertension, as well as for treating patients suffering from chronic hypertension.

The VEGF may be prepared for storage or administration by mixing the VEGF having the desired degree of purity with physiologically acceptable carriers, excipients, or stabilizers. Suitable carrier vehicles and their formulation, inclusive of other human proteins, for example, human serum albumin, are described, for example, in *Remington's Pharmaceutical Sciences*, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. Typically, an appropriate amount of a pharmaceutically-acceptable salt is used in the formulation to render the formulation isotonic. Examples of the carrier include buffers such as saline, Ringer's solution, and dextrose solution. The pH of the solution is preferably from about 5.0 to about 8.0. For example, if the VEGF is water soluble, it may be formulated in a buffer such as phosphate or other organic acid salt at a pH of about 7.0 to 8.0. If a VEGF is only partially soluble in water, it may be prepared as a microemulsion by formulating it with a nonionic surfactant such as Tween, Pluronic, or PEG, e.g., Tween 80, in an amount of 0.04-0.05% (w/v), to increase its solubility.

Further carriers include sustained release preparations which include the formation of microcapsular particles and implantable articles. Examples of sustained release preparations include, for example, semipermeable matrices of solid hydrophobic polymers, which matrices are in the form of shaped articles, e.g., films, liposomes or microparticles. A suitable material for this purpose is a polylactide, although other polymers of poly-(beta-hydroxycarboxylic acids), such as poly-D-(-)-3-hydroxybutyric acid [EP 133,988A], can be used. Other biodegradable polymers such as, for example, poly(lactones), poly(acetals), poly(orthoesters), or poly(ortho-carbonates) are also suitable.

For examples of sustained release compositions, see U.S. Patent No. 3,773,919, EP 58,481A, U.S. Patent No. 3,887,699, EP 158,277A, Canadian Patent No. 1176565, Sidman et al., *Biopolymers*, 22:547 (1983), and Langer et al., *Chem. Tech.*, 12:98 (1982).

It will be apparent to those persons skilled in the art that certain carriers may be more preferable depending upon, for instance, the route of administration and concentration of the VEGF being administered.

Optionally other ingredients may be added such as antioxidants, e.g., ascorbic acid; low molecular weight (less than about ten residues) polypeptides, e.g., polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as

polyvinylpyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates, including cellulose or its derivatives, glucose, mannose, or dextrans; chelating agents such as EDTA; and sugar alcohols such as mannitol or sorbitol. The use of excipients, carriers, stabilizers, or other additives may result in the formation of salts of the VEGF.

When selecting carriers, excipients, stabilizers, or other additives, the selected compound(s) and corresponding degradation products should be nontoxic and avoid aggravating the condition treated and/or symptoms thereof. This can be determined by routine screening in animal models of the target disorder or, if such models are unavailable, in normal animals.

The VEGF to be used for therapeutic administration should be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). The VEGF ordinarily will be stored in lyophilized form or as an aqueous solution.

Administration to a mammal may be accomplished by injection (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular) or by other methods such as infusion that ensure delivery to the bloodstream in an effective form. If the VEGF is to be used parenterally, therapeutic compositions containing the VEGF generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Generally, where the condition permits, one may formulate and dose the VEGF for site-specific delivery.

In one aspect, the VEGF of the invention may be administered by intravenous infusion at a dose of approximately up to 0.05 microgram/Kg/minute for about 4 hours on a daily schedule. Such VEGF may be administered at 48 hour or 72 hour intervals. Optionally, the VEGF may be administered intramuscularly or subcutaneously in a sustained release formulation at a dose of approximately 0.25 to about 2.5 mg/Kg, preferably, approximately 0.3 to about 1.0 mg/Kg. Still further, the VEGF may be administered to the mammal via a plasmid or viral vector to provide a sustained expression of the VEGF gene product to improve endothelial cell function or eNOS expression. Also contemplated is the use of VEGF in stent implantation. Local delivery of VEGF coated STENT provides for local eNOS upregulation and is beneficial to, for example, inhibit restenosis after balloon injury, since NO is a potent antithrombotic agent and has also been shown to inhibit smooth muscle cell (SMC) proliferation and restenosis.

The VEGF of the invention can also be used in a topical application for treating indications such as wound healing. When applied topically, VEGF can upregulate local eNOS and or iNOS production so as to enhance healing and prevent infection. When used in a topical application, the VEGF can be suitably combined with additives, such as carriers, adjuvants, stabilizers, or excipients. As described above, when selecting additives for admixture with a VEGF, additives should be pharmaceutically acceptable

and efficacious for their intended administration. Further, additives should not affect the activity of the VEGF. Examples of suitable topical formulations include ointments, creams, gels, or suspensions, with or without purified collagen. The compositions also may be impregnated into transdermal patches, plasters, and bandages, preferably in liquid or semi-liquid form.

A gel formulation having the desired viscosity may be prepared by mixing VEGF with a water-soluble polysaccharide, such as a cellulose derivative, or synthetic polymer, such as polyethylene glycol. The term "water soluble" as applied to the polysaccharides and polyethylene glycols is meant to include colloidal solutions and dispersions. In general, the solubility of, for example, cellulose derivatives is determined by the degree of substitution of ether groups, and the stabilizing derivatives useful herein should have a sufficient quantity of such ether groups per anhydroglucose unit in the cellulose chain to render the derivatives water soluble. A degree of ether substitution of at least 0.35 ether groups per anhydroglucose unit is generally sufficient. Additionally, the cellulose derivatives may be in the form of alkali metal salts, for example, Li, Na, K, or Cs salts.

Examples of suitable polysaccharides include, for example, cellulose derivatives such as etherified cellulose derivatives, including alkyl celluloses, hydroxyalkyl celluloses, and alkylhydroxyalkyl celluloses, for example, methylcellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose; starch and fractionated starch; agar; alginic acid and alginates; gum arabic; pullullan; agarose; carrageenan; dextrans; dextrans; fructans; inulin; mannans; xylans; arabinans; chitosans; glycogens; glucans; and synthetic biopolymers; as well as gums such as xanthan gum; guar gum; locust bean gum; gum arabic; tragacanth gum; and karaya gum; and derivatives and mixtures thereof. The preferred gelling agent herein is one that is inert to biological systems, nontoxic, simple to prepare, and not too runny or viscous, and will not destabilize the VEGF held within it.

Preferably the polysaccharide is an etherified cellulose derivative, more preferably one that is well defined, purified, and listed in USP, for example, methylcellulose and the hydroxyalkyl cellulose derivatives, such as hydroxypropyl cellulose, hydroxyethyl cellulose, and hydroxypropyl methylcellulose. Most preferred herein is methylcellulose. For example, a gel formulation comprising methylcellulose preferably comprises about 2-5% methylcellulose and 300-1000 mg of VEGF per milliliter of gel. More preferably, the gel formulation comprises about 3% methylcellulose.

The polyethylene glycol useful for a gel formulation is typically a mixture of low and high molecular weight polyethylene glycols to obtain the proper viscosity. For example, a mixture of a polyethylene glycol of molecular weight 400-600 with one of molecular weight 1500 would be effective for this purpose when mixed in the proper ratio to obtain a paste.

It is within the scope hereof to combine the VEGF therapy with other novel or conventional therapies (e.g., growth factors such as aFGF, bFGF, PDGF, IGF, NGF, HGF, anabolic steroids, EGF or TGF-beta). It is not necessary that such co-treatment drugs be included *per se* in the compositions of this invention, although this will be convenient where such drugs are proteinaceous. Such admixtures are suitably administered in the same manner as the VEGF.

The afore-described formulations and modes of administration may also be utilized to administer other molecules which modulate VEGF receptor activity, such as VEGF receptor selective variants or VEGF receptor antibodies. Effective dosages and schedules for such administration may be determined empirically, and making such determinations is within the skill in the art.

The following examples are offered for illustrative purposes only and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the Examples below were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, Virginia.

EXAMPLE 1

VEGF Up-Regulation of eNOS Expression

To study whether VEGF regulates eNOS expression, bovine adrenal cortex endothelial cells (ACE) cells were incubated with rhVEGF for 0-5 days. At the end of incubation, total cell lysates were prepared, and eNOS protein levels were determined by Western blot analysis.

Materials:

Recombinant human VEGF₁₆₅ (rhVEGF₁₆₅) was produced in *E. Coli* [see, Siemeister et al., *Biochem. Biophys. Res. Comm.*, 222:249-255 (1996); also available from R & D Systems]. The VEGF₁₁₀ heparin binding domain-deficient variant was made from VEGF₁₆₅ by limited proteolytic digestion with plasmin as described previously (Keyt et al., 1996, *J. Biol. Chem.*, 271:7788-7795).

VEGF receptor selective mutants FLT-sel (R82E/K84E/H86E, deficient in KDR binding) and KDR-sel (D63A/E64A/E67A, deficient in FLT-1 binding) were prepared using the Muta-Gene Phagemid *in vitro* mutagenesis kit as described previously (Keyt et al., 1996, *J. Bio. Chem.*, 271:4538-5646). The heterodimeric form of recombinant human hepatocyte growth factor (HGF) was produced in and isolated from Chinese hamster ovary cells as previously described (Shen et al., 1997, *Am. J. Physiol.*, 272:L1115-L1120).

Recombinant human fibroblast growth factor (FGF) basic, recombinant human placental growth factor (PLGF), recombinant human transforming growth factor (TGF- β 1, TGF- β 2), and recombinant human epidermal growth factor (EGF) were purchased from R&D Systems, Inc. (Minneapolis, MN). Monoclonal anti-eNOS antibody was purchased from BIOMOL (Plymouth Meeting, PA). Monoclonal anti-PKC antibodies were purchased from Transduction Lab (Lexington, KY). SU1498 was purchased from CALBIOCHEM (San Diego, CA). PP1, PD98059, staurosporine, herbimycin A, genistein, phorbol 12-myristate 13-acetate (PMA), wortmannin, Ly294002, L-N^G-Nitroarginine methyl ester (L-NAME) were ordered from BIOMOL (Plymouth Meeting, PA). Sodium nitroprusside (SNAP) was from Sigma (St. Louis, MO). NOSdetect Assay kit was purchased from STRATAGENE (La Jolla, CA). L-[2,3,4,5-³H]Arginine monohydrochloride was purchased from Amersham Pharmacia Biotech. Geneticin (G480) was obtained from Life Technologies (Gaithersburg, MD). All reagents were prepared as 1000X stock solution unless otherwise specified.

Cell Cultures:

Bovine adrenal cortex capillary endothelial cells (ACE) were prepared and maintained as previously described (Ferrara et.al., 1989 *Biochem. Biophys. Res. Commun.* 161:851-858). Briefly, cells were plated onto 6-well tissue culture plates (Costar) and grown in low glucose Dulbecco's modified Eagle's medium, supplemented with 2 mM L-Glutamate (Life Technologies), 10% bovine calf serum (HyClone Lab., Inc.) and 100 μ g/ml Penicillin/Streptomycin (Life Technologies). ACE cells were used between passages 4 and 8.

Porcine aorta endothelial (PAE) cells, and receptor transfected PAE cells (PAE/KDR and PAE/FLT-1) were provided by Napoleone Ferrara (Genentech, Inc.) and cultured in Ham's F-12 medium containing 10% FBS (for PAE), or plus 250 μ g/ml G480 (for PAE/KDR and PAE/FLT-1).

For drug treatment, cells were incubated in the medium containing 10% FBS supplemented with VEGF or other drugs as specified. Medium was changed every 24 hours.

Western blot detection of eNOS:

The methods for cell lysis and Western blot (WB) have been described in Shen et.al., 1998 *J. Biol. Chem.* 273:29979-29985. A monoclonal anti-eNOS antibody was used at 1:2500 to probe eNOS protein. A secondary antibody conjugated with horseradish peroxidase (1:2500) (Zymed) and an enhanced chemiluminescent kit (Amersham Pharmacia Biotech) were used to visualize the eNOS immunoreactive bands. Multiple exposures of films were obtained to determine the optimal exposure time. The protein bands were scanned by a densitometer and the relative intensities were quantified using ImageQuant software (Molecular Dynamics).

eNOS activity assay:

The eNOS activity in ACE cells treated with or without rhVEGF for 2 days was determined by measuring the formation of [³H]citrulline from [³H]arginine. Briefly, ACE cells were homogenized in a buffer containing 25

mM Tris-HCl, pH 7.4, 1 mM EDTA and 1 mM EGTA, and then subjected to microcentrifugation at 14,000 rpm for 5 minutes. 50 μ g of protein from the supernatants was incubated with 1 mM NADPH, 25 mM Tris-HCl, pH 7.4, 3 μ M tetrahydrobiopterin (BH₄), 1 μ M flavin adenine dinucleotide (FAD), 1 μ M flavin adenine mononucleotide, 0.1 μ Ci/ml of [³H]arginine, and other cofactors (calcium and calmodulin) provided in the assay kit (STRATAGENE) at 37°C for 45 minutes. The reaction was stopped with 50 mM HEPES, pH 5.5, 5 mM EDTA. Equilibrated resin, which binds to the arginine, was added to the reactions and then pipetted into spin cups. [³H]citrulline, which is ionically neutral at pH 5.5, flowed through the cups completely and was then quantitated by scintillation counting.

Confluent ACE cells were incubated with 500 pM rhVEGF. Total cell lysates were prepared as described above. Equal amounts of protein were denatured under reducing conditions and separated in a 10% Novex mini gel and transferred to PVDF membrane. eNOS protein signals were visualized by ECL and quantified using densitometry. The eNOS activity in cells treated with or without VEGF for 2 days was assayed using a NOS detection kit from Stratagene according to the manufacturer's protocol.

cGMP assay:

eNOS activity can also be determined by measuring the production of cyclic GMP (cGMP), a down-stream product following NO release as a result of eNOS activation. Papapetropoulis et al., *supra*. There are several commercial available cGMP assay kits, such as the one from BioMol. BioMol EIA cGMP kit is a competitive immunoassay for quantitative determination of cGMP in samples treated with 0.1M HCl. The kit uses a polyclonal antibody to cGMP to bind, in a competitive manner, the cGMP in sample or an alkaline phosphatase molecule which has cGMP covalently attached to it. After incubation, the excess reagents are washed away and substrate added. Then enzyme reaction is stopped and the yellow color generated is read on a microplate reader at 405nm. The intensity of the bound yellow color is inversely proportional to the concentration of cGMP in samples.

Results:

VEGF induced eNOS up-regulation in a dose-dependent manner with the maximal increase occurring following incubation with 500 pM VEGF (data not shown). Figure 1A shows the results of a time-course experiment, indicating that prolonged VEGF treatment induced a transient increase in eNOS expression. The peak expression (5.5 fold) was observed at 2 days (48 hours) post exposure to 500 pM VEGF (Figure 1B).

In addition, eNOS activity in cell lysates from cells incubated with or without VEGF for 0-60 min or 2 days was measured using a NOS detection kit. Figure 1C shows that eNOS activity in VEGF-treated cells was about 5 fold greater than that in untreated control cells, which was proportional to the increased eNOS protein levels (5.5 fold). Figure 1D shows that acute

VEGF treatment (0-60 min) resulted in a time-dependent increase in eNOS activity.

The total NOS activity measured in ACE cells likely represents eNOS activity because (1) the assay measured Ca^{+2} dependent NOS activity and (2) there was no detectable eNOS proteins by Western blot in either VEGF treated or untreated cells (data not shown). Together, these data demonstrate that VEGF increases both eNOS expression and activity in cultured endothelial cells.

Example 2

Negative Feedback of NO on eNOS Expression

The role of nitric oxide in VEGF-induced eNOS expression was investigated by co-incubation of VEGF with L-NAME (2mM), an eNOS inhibitor, with SNAP, a nitric oxide donor, or with L-arginine, the substrate of eNOS. ACE cells were incubated with VEGF alone or in combination with an eNOS inhibitor, L-NAME. eNOS protein was detected by Western blot. The materials and methods used in this study were as described above for Example 1.

Figure 2 shows that VEGF alone induced a time-dependent but transient increase in eNOS expression with a maximal effect at 2 days post-exposure.

In contrast, co-incubation of L-NAME with VEGF resulted in a sustained increase in eNOS expression, whereas NO donor, SNAP, prevented both transient and sustained increase in eNOS (Figure 2). These results suggest that nitric oxide produced by eNOS in endothelial cells in culture or from exogenous nitric oxide donors may have a negative feedback on eNOS expression.

Example 3

KDR Receptor Activation is Required for VEGF-induced eNOS Expression

Several approaches were used to determine which VEGF receptor was involved in VEGF-induced eNOS expression. First, VEGF receptor-selective variants which bind preferentially to either one of the VEGF receptors, KDR or FLT-1-, at equal concentrations, were incubated with ACE cells for two days. The specific agents used in this study and shown in Figure 3A were: lane 1, control; lane 2, VEGF_{165} ; lane 3, VEGF_{110} ; lane 4, KDR-sel; lane 5, FLT-sel; lane 6, PLGF. eNOS protein was detected using the methods described for Example 1. The materials and methods used in this study were as described above for Example 1.

Figure 3A shows that both VEGF_{165} and VEGF_{110} , a heparin binding domain-deficient mutant with normal binding to KDR and FLT-1, induced a similar degree of eNOS expression. The KDR selective variant (KDR-sel) binds to KDR receptor normally but with reduced binding to FLT-1. The KDR-selective variant up-regulated eNOS expression, whereas the FLT-1 selective binding variant (FLT-sel) failed to do so. Placental growth factor (PLGF), which is known to only bind to FLT-1 receptor, had no effect on eNOS expression.

These data suggest that the KDR receptor was the dominant receptor involved in VEGF-induced eNOS expression.

The involvement of KDR receptor in eNOS up-regulation was further confirmed by experiments in which receptor transfected porcine aorta endothelial (PAE) cells were used. PAE, PAE/KDR and PAE/Flt-1 cells were incubated with or without 500 pM rhVEGF for 2 days. eNOS was detected as described above for Example 1. Other materials and methods used in this study were as described above for Example 1.

Data shown in Figure 3B demonstrate that VEGF was able to induce eNOS up-regulation only in KDR-transfected PAE (PAE/KDR) cells, and not in FLT-1 transfected PAE (PAE/FLT) cells.

Activation of KDR receptor tyrosine kinase was shown to be required for eNOS up-regulation. Co-incubation of VEGF with a selective KDR tyrosine kinase inhibitor, SU-149g caused a dose dependent inhibition of eNOS expression. (Figure 3C)

The materials and methods used in this study were as described above for Example 1. Confluent ACE cells were co-incubated with SU1498 in combination with 500 pM rhVEGF for 2 days. The data shown is representative of 3 independent experiments.

At high concentration (100 μ M), SU-1498 completely blocked VEGF-induced eNOS up-regulation. Taken together, these data demonstrate that KDR receptor activation is required for VEGF-induced eNOS expression.

Example 4

Inhibition of Tyrosine Kinase and/or PKC Inhibits VEGF-induced eNOS Expression

Various inhibitors were tested to investigate the role of down-stream signaling molecules following receptor activation in VEGF-induced eNOS expression. The materials and methods used in this study were as described above for Example 1. ACE cells were treated with specific tyrosine kinase inhibitors in combination with 500 pM rhVEGF for 2 days. eNOS protein was detected by Western blot.

Specific agents used and shown in Figure 4 are: Lane 1, control (no VEGF or inhibitors); lane 2, VEGF (500 pM); lane 3, VEGF and herbimycin A ("Her"; 2 μ M); lane 4, VEGF + PP1 (10 μ M); lane 5, VEGF and staurosporin ("Sta"; 500 nM); lane 6, VEGF and GFX (GF109203X, 10 μ M); lane 7, VEGF and Calphostin C ("Cal", 10 μ M); lane 8, VEGF and chelerythrine chloride ("che", 10 μ M); lane 9, VEGF and Go6976 ("Go", 10 μ M); lane 10, VEGF and Rotterlin Mallotoxin ("Rot", 50 μ M); lane 11, VEGF and H8 (25 μ M); lane 12, VEGF and H89 (25 μ M). The data shown is representative of 3 independent experiments.

The data in Figure 4A shows that inhibition of tyrosine kinase activity with genistein or herbimycin A, an irreversible and selective inhibitor of tyrosine kinase, blocked VEGF-induced eNOS expression.

Furthermore, inhibition of PLC-gamma, the upstream signal molecule of PKC, with PP1 also blocked eNOS expression. Inhibition of PI3-K with Wortmannin had no significant effect on eNOS expression. Figure 4B shows the respective quantitation of eNOS levels using a densitometer.

5 To examine the role of PKC isoforms and activation in VEGF-induced eNOS up-regulation, ACE cells were treated with rhVEGF (500 pM) for 10 minutes. Cytosolic and membrane fractions were prepared to investigate the re-distribution of PKC isoforms. VEGF induced a rapid re-distribution of PKC-alpha, -gamma, and -epsilon. (See Figure 5A). In addition, VEGF
10 treatment time-dependently increased PKC activity (Figure 5B).

Incubation with PMA, a potent PKC activator, resulted in a time-dependent (Figure 5C) and dose-dependent (data not shown) increase in eNOS levels. These data imply an important role for PKC activation and signaling in VEGF-induced eNOS expression.

Example 5

Effect of Other Angiogenic Factors on eNOS Expression

The effect of several other angiogenic factors including FGF, HGF, EGF, TGF-beta on eNOS expression was also studied. ACE cells were treated with equal molar concentrations (500 pM) of VEGF, HGF, FGF, EGF, TGF-beta1 or TGF-beta2 for 2 days. The materials and methods used in this study were as described above for Example 1.

Figure 6 shows that in addition to VEGF, HGF is also capable of increasing eNOS expression, whereas FGF, EGF had no significant effect on eNOS expression. TGF-beta1 and TGF-beta2 reduced endogenous eNOS expression. These data indicate that VEGF and HGF are distinct from the other angiogenic growth factors in their ability to increase eNOS expression *in vitro*.

EXAMPLE 6

Selection of KDR-Specific VEGF Variants

To generate KDR-specific variants, two phage libraries were constructed in which residues of VEGF(1-109) found to be important for Flt-1 binding but not KDR binding were randomly mutated.

Phagemid Construction

To construct the phage libraries, a phagemid vector having cDNA encoding residues 1-109 of VEGF was first produced. Phagemid vector pB2105 (Genentech, Inc.) was produced by PCR amplification of the cDNA encoding residues 1-109 of VEGF, using primers that allowed subsequent ligation of Nsi I / Xba I restriction fragment into the phagemid vector, pHGHam-g3 (Genentech, Inc.). This introduced an amber codon immediately following residue 109 and fused the VEGF 1-109 cDNA to the C-terminal half of gIII encompassing residues 249 through 406.

5

NNS sequences:

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Synthesis of Heteroduplex DNA

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First, the oligonucleotides described above were 5'-phosphorylated.

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The DNA was purified by ethanol precipitation and resuspended in 15 μ l of water.

E. coli Electroporation

The library phage were produced in a supressor strain of *E. coli* known as *E. coli* XL1-blue (Stratagene, LaJolla, CA) by *E. coli* electroporation. For electroporation, purified heteroduplex DNA first was chilled in a 0.2-cm gap electroporation cuvet on ice, and a 100 μ l aliquot of electrocompetent *E. coli* XL1-blue was thawed on ice. The *E. coli* cells were added to the DNA and mixed by pipetting several times.

The mixture was transferred to the cuvet and electroporated using a Gene Pulser (Bio-rad, Hercules, CA) with the following settings: 2.5 kV field strength, 200 ohms resistance, and 25 mF capacitance. Immediately thereafter, 1 ml of SOC media (5 g bacto-yeast extract, 20 g bacto-tryptone, 0.5 g NaCl, 0.2 g KCl; add water to 1 liter and adjust pH to 7.0 with NaOH; autoclave; then add 5 mL of autoclaved 2 M $MgCl_2$ and 20 mL of filter sterilized 1 M glucose) was added and the mixture was transferred to a sterile culture tube and grown for 30 minutes at 37°C with shaking.

To determine the library diversity, serial dilutions were plated on 2YT (10 g bacto-yeast extract, 16 g bacto-tryptone, 5 g NaCl; add water to 1 liter and adjust pH to 7.0 with NaOH; autoclaved) plates (supplemented with 50 μ g/ml ampicillin). Additionally, the culture was transferred to a 250-ml baffled flask containing 25 ml 2YT, 25 mg/ml ampicillin, M13-VCS (10^{10} pfu/mL) (Stratagene, LaJolla, CA), and incubated overnight at 37°C with shaking.

The culture was then centrifuged for 10 minutes at 10 krpm, 2°C, in a Sorvall GSA rotor (16000g). The supernatant was transferred to a fresh tube and 1/5 volume of PEG-NaCl solution (200 g/L PEG-8000, 146 g/L NaCl; autoclaved) was added to precipitate the phage. The supernatant/PEG-NaCl solution was incubated for 5 minutes at room temperature and centrifuged again to obtain a phage pellet.

The supernatant was decanted and discarded. The phage pellet was recentrifuged briefly and the remaining supernatant was removed and discarded. The phage pellet was resuspended in 1/20 volume of PBT buffer (PBS, 0.2% BSA, 0.1% Tween 20), and insoluble matter was removed and discarded by centrifuging the resuspended pellet for 5 minutes at 15 krpm, 2°C, in a SS-34 rotor (27000g). The remaining supernatant contained the phage.

The supernatant was saved and used for sorting VEGF variants by their binding affinities. By producing the phage in a suppressor strain of *E. coli*, VEGF (1-109) variants-gIII fusion protein were expressed and displayed on the phage surface, allowing the phage to bind to KDR and/or Flt-1 receptors.

Affinity Sorting of the Libraries

Each library was sorted for binding to KDR (1-3) monomer using a competitive binding technique similar to a method used by H. Jin, *J. Clin. Invest.*, 98: 969 (1996), and shown to be useful for generating receptor-selective variants.

To conduct the competitive binding technique, each library was sorted for binding to immobilized KDR (1-3) monomer (Genentech, South San Francisco, California) in the presence of a high concentration (100 nM) of competing Flt-1 (1-3) monomer (Genentech, Inc.) in solution. This was accomplished by first coating Maxisorp immunoplate wells (Nalge Nunc International, Rochester, New York) with 80 μ l per well of 2-5 μ g/ml of KDR (1-3) monomer in coating buffer (50mM sodium carbonate at pH 9.6) and incubating overnight at 4°C. The number of wells required depends on the diversity of the library. The coating solution was removed and blocked for 1 hour with 200 μ l of 0.2% BSA in PBS. At the same time, an equal number of uncoated wells were blocked as a negative control.

The wells were washed eight times with PT buffer (PBS, 0.05% Tween 20) to remove the block buffer. Aliquots of 100 μ l of library phage solution (10^{12} phage/ml) in PBT buffer (PBS, 0.2% BSA, 0.1% Tween 20) were then added to each of the coated and uncoated wells. The Flt-1 (1-3) monomer was added with the phage solution. The wells were incubated at room temperature for 2 hours with gentle shaking.

The wells were then washed 10 times with PT buffer (PBS, 0.05% Tween 20) to remove the phage solution and any Flt-1-bound phage. KDR-bound phage was eluted from the wells by incubating the wells with 100 μ l of 0.2 M glycine at pH 2 for 5 minutes at room temperature. To collect the KDR-bound phage, the glycine solution was transferred to an eppendorf tube and neutralized with 1.0 M Tris-HCl at pH 8.0.

The KDR-bound phage were then repropagated by adding half of the eluted phage solution to 10 volumes of actively growing *E. coli* XL1-blue ($OD_{600} < 10$) and incubating for 30 minutes at 37°C with shaking. The serial dilutions of the culture were then plated on 2YT/amp plates (2YT being supplemented with 50 mg/ml ampicillin) to determine the number of phage eluted. This was determined for both the wells coated with KDR (1-3) monomer and the uncoated control wells.

The culture from the plates was transferred to 10 volumes of 2YT/amp/VCS (2 YT being supplemented with 50 mg/ml ampicillin and 10^{10} pfu/ml M13-VCS) and incubated overnight at 37°C with shaking. The phage were then isolated.

The phage that were repropagated were again sorted for binding to immobilized KDR (1-3) monomer in the presence of a high concentration (100 nM) of competing Flt-1 (1-3) monomer, followed by washing away the Flt-1-bound phage and repropagating the KDR-bound phage. The affinity sort

procedure was monitored by calculating the enrichment ratio and was repeated until the enrichment ratio reached a maximum (about 5 to 6 sorting cycles).

The enrichment ratio is the number of phage eluted from a well coated with KDR (1-3) monomer divided by the number of phage binding to an uncoated control well. A ratio greater than one is usually indicative of phage binding specifically to the KDR (1-3) protein, thereby indicating resistance to binding to added Flt-1 (1-3) monomer. When the enrichment ratio reached a maximum, individual clones were analyzed for specific binding.

Phage ELISA

Specific binding of phage having VEGF 1-109 variant-gIII protein on its surface to the KDR (1-3) monomer was measured using a phage ELISA according to Muller et al., *PNAS*, 94: 7192 (1997). For the phage ELISA, microtiter plates (Maxisorp, Nunc-Immunoplate, Nalge Nunc International, Rochester, New York) were coated with purified KDR (1-3) monomer or Flt-1 (1-3) monomer (5ug/ml) in 50mM sodium carbonate at pH 9.6 and incubated at 4 °C overnight. The plates were blocked with 0.5% BSA. Next, serial dilutions of VEGF 1-109 variants together with a subsaturating concentration of competing receptor (KDR (1-3) monomer or Flt-1 (1-3) monomer) were added to wells in 100 ul of binding buffer (PBS, 0.5% Tween20, 0.5% BSA). After equilibrium, the plates were washed, and the bound phagemid were stained with horseradish peroxidase-conjugated anti-M13 antibody (Pharmacia Biotech, Piscataway, NJ), following manufacturer instructions. Affinities (EC50) were calculated as the concentration of competing receptor that resulted in half-maximal phagemid binding.

The sequences of VEGF 1-109 variants which were obtained from the affinity sorting and which showed resistance to Flt-1 (1-3) monomer were determined from the sequence of the phagemid cDNA.

Purification of VEGF 1-109 Variants

VEGF 1-109 variant proteins were isolated as retractile bodies from the shake flask culture of *E. coli* (27c7). The refolding of the mutant proteins was performed as described by Yihai et al., *J. Biol. Chem.*, 271: 3154-3162 (1996). The variants were mixed and unfolded with 6 M guanidine HCL plus 1 mM oxidized glutathione at pH 6, and dialyzed against 10 volumes of 2 M urea with 2 mM reduced glutathione and 0.5 mM of oxidized glutathione in 20 mM Tris-HCL at pH 8 for 10 hours. Urea was removed by dialyzing slowly against 20 volumes of 20mM Tris-HCL (pH 8) overnight at 4°C. Each of the variants was purified further by anion exchange (Pharmacia HiTrap Q, 1ml) (Pharmacia Biotech, Piscataway, NJ), to remove traces of misfolded monomer. The identity of the resulting pure variants was confirmed by SDS-PAGE and mass spectrometry.

Table 2 shows the VEGF variant identifier name, the amino acid substitutions introduced, and the codon encoding the respective substituted amino acids. The asterisk (*) next to certain variant identifiers (such as

LK-VRB-1s) indicates various VEGF variants which demonstrated particularly preferred binding affinities and/or biological activities. The variant identifiers which contain an "s" (such as LK-VRB-1s) indicate VEGF variant polypeptides which consisted of the 1-109 truncated form of VEGF and contained the recited mutations provided in the Table. The variant identifiers which contain an "f" (such as LK-VRB-1f) indicate VEGF variant polypeptides which consisted of the full length 1-165 form of VEGF and contained the recited mutations provided in the Table. The naming and identification of the mutations in the variant sequences is in accord with naming convention. For example, for the first entry in Table 2, the mutation is referred to as "M18E". This means that the 18 position of the native VEGF sequence (using the numbering in the amino acid sequence for native human VEGF as reported in Leung *et al.*, *supra* and Houck *et al.*, *supra*) was mutated so that the native methionine (M) at that position was substituted with a glutamic acid (E) residue to prepare the VEGF variant. The column in Table 2 referred to as "Nucleotide Sequence" provides the respective codons coding (5' → 3') for each of the respective amino acid mutations. For example, for the first entry in Table 2, the M18E mutation is coded by the codon "GAG".

TABLE 2
VEGF Variants and Corresponding Mutations

| Variant Identifier | Amino Acid Mutation | Nucleotide Sequence |
|--------------------|------------------------------|-------------------------|
| LK-VRB-1s* | M18E/Y21L/Q22R/Y25S | GAG/CTC/CGG/AGC |
| LK-VRB-2s* | D63S/G65M/L66R | AGC/ATG/CGC |
| LK-VRB-3s | F17I/M18E/Y21F/Q22K/Y25S | ATT/GAG/TTC/AAG/ AGC |
| LK-VRB-4s | F17I/M18E/Y21F/Q22E/Y25I | ATC/GAG/TTC/GAG/ CAC |
| LK-VRB-5s | D63S/L66R | AAG/CAG |
| LK-VRB-6s | D63S/G65A/L66T | AAG/GGC/ATG |
| LK-VRB-7s* | M18E/D63S/G65M/L66R | GAG/AGC/ATG/CGC |
| LK-VRB-8s* | Y21L/D63S/G65M/L66R | CTC/AGC/ATG/CGC |
| LK-VRB-9s | Q22R/D63S/G65M/L66R | CGG/AGC/ATG/CGC |
| LK-VRB-10s | Y25S/D63S/G65M/L66R | AGC/AGC/ATG/CGC |
| LK-VRB-11s | M18E/Y21L/ D63S/G65M/L66R | GAG/CTC/ AGC/ATG/CGC |
| LK-VRB-12s | M18E/Q22R/ D63S/G65M/L66R | GAG/CGG/ AGC/ATG/CGC |
| LK-VRB-13s | M18E/Y25S/ D63S/G65M/L66R | GAG/AGC/ AGC/ATG/CGC |
| LK-VRB-14s | Y21L/Q22R/ D63S/G65M/L66R | CTC/CGG/ AGC/ATG/CGC |

| Variant Identifier | Amino Acid Mutation | Nucleotide Sequence |
|--------------------|--|---------------------------------|
| LK-VRB-15s | Y21L/Y25S/ D63S/G65M/L66R | CTC/AGC/ AGC/ATG/CGC |
| LK-VRB-16s | Q22R/Y25S/ D63S/G65M/L66R | CGG/AGC/ AGC/ATG/CGC |
| LK-VRB-17s | M18E/Y21L/Q22R/ D63S/G65M/L66R | GAG/CTC/GAG/ AGC/ATG/CGC |
| LK-VRB-18s | M18E/Q22R/Y25S/ D63S/G65M/L66R | GAG/CGG/AGC/ AGC/ATG/CGC |
| LK-VRB-19s | M18E/Q22R/Y25S/ D63S/G65M/L66R | GAG/CGG/AGC/ AGC/ATG/CGC |
| LK-VRB-20s | Y21L/Q22R/Y25S/ D63S/G65M/L66R | CTC/CGG/AGC/ AGC/ATG/CGC |
| LK-VRB-21s | D63S/ M18E/Y21L/Q22R/Y25S | TCC/ GAG/CTC/CGG/AGC |
| LK-VRB-22s | G65M/ M18E/Y21L/Q22R/Y25S | ATG/ GAG/CTC/CGG/AGC |
| LK-VRB-23s | L66R/ M18E/Y21L/Q22R/Y25S | AGG/ GAG/CTC/CGG/AGC |
| LK-VRB-24s | D63S/G65M/ M18E/Y21L/Q22R/Y25S | TCC/ATG/ GAG/CTC/CGG/AGC |
| LK-VRB-25s | D63S/L66R/ M18E/Y21L/Q22R/Y25S | TCC/AGG/ GAG/CTC/CGG/AGC |
| LK-VRB-26s | G65M/L66R/ M18E/Y21L/Q22R/Y25S | ATG/AGG/ GAG/CTC/CGG/AGC |
| LK-VRB-27s | M18E/Y21L/Q22R/Y25S/D63S /G65M/L66R | GAG/CTC/CGG/AGC/ AGC/ATG/CGC |
| LK-VRB-1f | M18E/Y21L/Q22R/Y25S | GAG/CTC/CGG/AGC |
| LK-VRB-2f | D63S/G65M/L66R | AGC/ATG/CGC |

EXAMPLE 7

Binding of VEGF Variants to KDR Receptor

5 The binding of VEGF (1-109) variants and VEGF165 variants (described in Example 6) to KDR receptor was evaluated by measuring the ability of the variants to inhibit binding of biotinylated native VEGF (8-109) to KDR receptor. The VEGF variants evaluated contained the mutations shown in Table 2.

10 Receptor binding assays were performed in 96-well immunoplates (Maxisorp, Nunc-Immunoplate, Nalge Nunc International, Rochester, New York).

Each well was coated with 100 μ l of a solution containing 8 μ g/ml of a monoclonal antibody to KDR known as MAKD5 (Genentech, South San Francisco, California) in 50 mM carbonate buffer at pH 9.6 and incubated at 4°C

overnight. The supernatant was discarded, the wells were washed three times in washing buffer (0.05% Tween 20 in PBS), and the plate was blocked (150 μ l per well) with block buffer (0.5% BSA, 0.01% thimerosal in PBS) at room temperature for one hour. The supernatant was discarded, and the wells were washed.

Serially diluted native VEGF(8-109), native VEGF (1-165), native VEGF (1-109) variants, or VEGF165 variants (0.16-168 nM in monomer) were incubated with biotinylated native VEGF (8-109) (84 nM) and KDR (1-3) (1 μ g/ml) for 2 hours at room temperature in assay buffer (0.5% BSA, 0.05% Tween 20 in PBS). Aliquots of this mixture (100 μ l) were added to the precoated microtiter wells and the plate was incubated for 1 hour at room temperature.

The complex of KDR (1-3) and biotinylated native VEGF that was bound to the microtiter plate was detected by incubating the wells with peroxidase-labeled streptavidin (0.2 mg/ml, Sigma, St. Louis, Missouri) for 30 minutes at room temperature. The wells were then incubated with 3, 3', 5, 5'-tetramethyl benzidine (0.2 gram/liter; Kirkegaard & Perry Laboratories, Gaithersburg, Maryland) for about 10 minutes at room temperature. Absorbance was read at 450 nm on a Vmax plate reader (Molecular Devices, Menlo Park, California).

Titration curves were fit with a four-parameter nonlinear regression curve-fitting program (KaleidaGraph, Synergy Software, Reading, Pennsylvania). Concentrations of VEGF variants corresponding to the midpoint absorbance of the titration curve of the native VEGF (8-109) were calculated and then divided by the concentration of the native VEGF corresponding to the midpoint absorbance of the native VEGF titration curve. (See Figure 7)

The binding affinities determined for the VEGF (1-109) variants and VEGF165 variants are shown in Table 3. Many of the VEGF variants exhibited binding to KDR receptor that was within about two-fold of the binding of native VEGF (8-109).

EXAMPLE 8

Binding of VEGF Variants to Flt-1 Receptor

The binding of the VEGF (1-109) variants and VEGF165 variants (described in Example 6) to Flt-1 receptor was evaluated by measuring the ability of the variants to inhibit binding of biotinylated native VEGF (8-109) to Flt-1 receptor. The VEGF variants evaluated contained the mutations shown in Table 2.

Receptor binding assays were performed in 96-well immunoplates (Maxisorp, Nunc-Immunoplate, Nalge Nunc International, Rochester, New York).

Each well was coated with 100 μ l of a solution containing 2 μ g/ml of rabbit F(ab')₂ to human IgG Fc (Jackson ImmunoResearch, West Grove, Pennsylvania) in 50 mM carbonate buffer at pH 9.6 and incubated at 4°C overnight. The

supernatant was then discarded, the wells were washed three times in washing buffer (0.05% Tween 20 in PBS), and the plate was blocked (150 μ l per well) with block buffer (0.5% BSA, 0.01% thimerosal in PBS) at room temperature for one hour. The supernatant was discarded, and the wells were washed.

5 The wells were filled with 100 μ l of a solution containing Flt-IgG (a chimeric Flt-human Fc molecule) at 50 ng/ml in assay buffer (0.5% BSA, 0.05% Tween 20 in PBS). The wells were incubated at room temperature for 1 hour and then washed three times in wash buffer (0.05% Tween 20 in PBS).

Serially diluted native VEGF(8-109), native VEGF165, VEGF (1-109)
10 variants, or VEGF165 variants (0.03-33 nM in monomer) were mixed with biotinylated native VEGF (8-109) (0.21 nM) or biotinylated native VEGF165 (0.66 nM). Aliquots of the mixture (100 μ l) were added to the precoated microtiter wells and the plate was incubated for 2 hours at room temperature. The complex of Flt-IgG and biotinylated native VEGF that was
15 bound to the microtiter plate was detected by incubating the wells with peroxidase-labeled streptavidin (0.2 mg/ml, Sigma, St. Louis, Missouri) for 30 minutes at room temperature. The wells were then incubated with 3, 3', 5, 5'-tetramethyl benzidine (0.2 g/liter, Kirkegaard & Perry Laboratories, Gaithersburg, Maryland) for about 10 minutes at room temperature.
20 Absorbance was read at 450 nm on a Vmax plate reader (Molecular Devices, Menlo Park, California).

Titration curves were fit with a four-parameter nonlinear regression curve-fitting program (KaleidaGraph, Synergy Software, Reading, Pennsylvania). Concentrations of VEGF variants corresponding to the
25 midpoint absorbance of the titration curve of the native VEGF (8-109) were calculated and then divided by the concentration of the native VEGF corresponding to the midpoint absorbance of the native VEGF titration curve.

The binding affinities determined for the VEGF (1-109) variants and VEGF165 variants are shown in Table 3. Many of the VEGF variants exhibited
30 binding to Flt-1 receptor that was more than 2,000-fold less than the binding of native VEGF (8-109). The relative binding affinity data reported in Table 3 for certain VEGF variants (for instance, LK-VRB-7s* and LK-VRB-8s*) to FLT-1 receptor is not reported in nM values since the amount of detectable binding was beyond the sensitivity of the ELISA assay.

35

TABLE 3
Binding of VEGF Variants to KDR Receptor
and FLT-1 Receptor

| Variant Identifier | Relative Binding Affinity | |
|--------------------|---------------------------|----------------|
| | KDR Receptor | FLT-1 Receptor |
| LK-VRB-1s* | 1 nM/1 | 2700 nM/6000 |
| LK-VRB-2s* | 1 nM/1 | >400 nM/>1000 |
| LK-VRB-3s | 1 nM/1 | 170 nM/400 |
| LK-VRB-4s | 1 nM/1 | 100 nM/200 |
| LK-VRB-5s | 1 nM/1 | 233 nM/550 |
| LK-VRB-6s | 0.5 nM/0.5 | 4 nM/10 |
| LK-VRB-7s* | 1 nM/1 | />15000 |
| LK-VRB-8s* | 0.5 nM/0.5 | />21000 |
| LK-VRB-9s | 0.5 nM/0.5 | /300 |
| LK-VRB-10s | 0.5 nM/0.5 | />2400 |
| LK-VRB-11s | 2 nM/2 | />14000 |
| LK-VRB-12s | 0.4 nM/0.4 | />5600 |
| LK-VRB-13s | 14 nM/14 | />14000 |
| LK-VRB-14s | 0.5 nM/0.5 | />2900 |
| LK-VRB-15s | 2 nM/2 | />21000 |
| LK-VRB-16s | 0.6 nM/0.6 | />1400 |
| LK-VRB-17s | 3 nM/3 | />1900 |
| LK-VRB-18s | 130 nM/130 | />3900 |
| LK-VRB-19s | 7 nM/7 | />35000 |
| LK-VRB-20s | 2 nM/2 | />10000 |
| LK-VRB-21s | 3 nM/3 | />5600 |
| LK-VRB-22s | 4 nM/4 | />30 |
| LK-VRB-23s | 11 nM/11 | />8500 |
| LK-VRB-24s | 10 nM/10 | />18000 |
| LK-VRB-25s | 4 nM/4 | />12000 |

| Variant Identifier | Relative Binding Affinity | |
|-----------------------------|---------------------------|----------------|
| | KDR Receptor | FLT-1 Receptor |
| LK-VRB-26s | 23 nM/23 | />25000 |
| LK-VRB-2f | 1 nM/1 | 19 nM/70 |
| Compare Native VEGF (8-109) | 1 nM/1 | 0.42 nM/1 |

EXAMPLE 9

Induction of KDR Receptor Phosphorylation by VEGF (1-109) Variants

To determine the activity of the VEGF variants, the ability of the variants to induce phosphorylation of the KDR receptor was measured in a KIRA assay. The VEGF variants evaluated contained the mutations found in Table 2. Specifically, the following VEGF (1-109) variants were studied: LK-VRB-1s*; LK-VRB-2s*; LK-VRB-3s; LK-VRB-4s; LK-VRB-5s; and LK-VRB-6s.

Serially diluted VEGF (1-109) variants (0.01-10 nM) were added to CHO cells that express the KDR receptor with a gD tag at the N-terminus (Genentech, South San Francisco, California). Cells were lysed by 0.5% Triton-X100, 150 mM NaCl, 50 mM Hepes at pH 7.2, and phosphorylated gD-KDR receptor in the lysate was quantified by conducting an ELISA.

For the ELISA, 96-well immunoplates (Maxisorp, Nunc-Immunoplate, Nalge Nunc International, Rochester, New York) were used. Each well was coated with 100 μ l of a solution containing 1 μ g/ml of a mouse monoclonal antibody to gD known as 3C8 (Genentech, South San Francisco, California) in 50 mM carbonate buffer at pH 9.6 and incubated overnight at 4°C. The supernatant was discarded, the wells were washed three times in washing buffer (0.05% Tween 20 in PBS), and the plate was blocked (150 μ l per well) in block buffer (0.5% BSA, 0.01% thimerosal in PBS) for 1 hour at room temperature. The supernatant was then discarded, and the wells were washed.

Aliquots of the lysate (100 μ l) were added to the precoated wells and incubated for 2 hours at room temperature. The phosphorylated gD-KDR receptor was detected by incubating the wells with biotinylated monoclonal antibody to phosphotyrosine known as 4G10 (0.05 mg/ml) (Upstate Biotechnology, Lake Placid, New York) for 2 hours at room temperature followed by incubating the wells with peroxidase-labeled streptavidin (0.2 mg/ml, Sigma, St. Louis, Missouri) for 1 hour at room temperature. The wells were then incubated with 3, 3', 5, 5'-tetramethyl benzidine (0.2 g/liter, Kirkegaard & Perry Laboratories, Gaithersburg, Maryland) for about 15-20 minutes at room temperature. Absorbance was read at 450 nm on a Vmax plate reader (Molecular Devices, Menlo Park, California).

Titration curves were fit with a four-parameter nonlinear regression curve-fitting program (KaleidaGraph, Synergy Software, Reading, Pennsylvania). Concentrations of VEGF variants corresponding to the midpoint absorbance of the titration curve of the native VEGF (8-109) were calculated and then divided by the concentration of the native VEGF corresponding to the midpoint absorbance of the native VEGF titration curve. (Figure 8)

The phosphorylation-inducing activity of the VEGF variants are provided in Table 4. The VEGF variants generally exhibited phosphorylation-inducing activity that was within two-fold of the activity of native VEGF (8-109).

TABLE 4
Induction of KDR Receptor Phosphorylation
By VEGF (1-109) Variants

| Variant Identifier | Phosphorylation-Inducing Activity |
|-----------------------------|-----------------------------------|
| LK-VRB-1s* | 1 nM/0.5 |
| LK-VRB-2s* | 2 nM/1 |
| LK-VRB-3s | 2 nM/1 |
| LK-VRB-4s | 1 nM/0.5 |
| LK-VRB-5s | 1 nM/0.5 |
| LK-VRB-6s | 1 nM/0.5 |
| Compare Native VEGF (8-109) | 2 nM/1 |

EXAMPLE 10

Endothelial Cell Proliferation Assay

The mitogenic activity of VEGF (1-109) or VEGF165 variants (as well as one VEGF165 variant, LK-VRB-2f) was determined by using human umbilical vein endothelial cells (HUVEC) (Cell Systems, Kirkland, Washington) as target cells. The VEGF variants evaluated contained the mutations in Table 2. Specifically, the following VEGF (1-109) variants were studied: LK-VRB-1s*; LK-VRB-2s*; LK-VRB-7s*; and LK-VRB-8s*.

HUVEC is a primary cell line that is maintained and grown with growth factors such as acidic FGF in CS-C Complete Growth media (Cell Systems, Kirkland, Washington). To prepare for the assay, an early passage (less than five passages) of the cells was washed and seeded in 96-well plates (3000 cells in 100 μ l per well) and fasted in CS-C media without any growth factors but supplemented with 2% Diafiltered Fetal Bovine Serum (GibcoBRL, Gaithersburg, MD) for 24 hours at 37°C with 5% CO₂ incubator before replacing with fresh fasting media. VEGF variants at several concentrations (about 10 nM to 0.01 nM) diluted in the same fasting media

were added to the wells to bring the volume to 150 μ l per well and incubated for 18 hours.

To measure the DNA synthesis induced by the VEGF variants, ^3H -thymidine (Amersham Life Science, Arlington Heights, IL) was added to each well at 0.5 μCi per well and incubated for another 24 hours for the cells to take up the radioactivity. The cells were then harvested onto another 96-well filter plate and the excess label was washed off before loading the plates on the Topcount (Packard, Meriden, Connecticut).

The cells were counted by Topcount. The measured counts per minute (CPM) were plotted against the concentration of individual variants to compare their activities. (Figure 9)

The cell proliferation capabilities of the VEGF variants are shown in Table 5. The VEGF variants generally exhibited cell proliferation capability that was within two-fold of the capability of native VEGF (8-109).

Table 5
Mitogenic Activity of VEGF (1-109) Variants

| Variant Identifier | Endothelial Cell Proliferation Activity |
|-----------------------------|---|
| LK-VRB-1s* | 0.1 nM/0.2 |
| LK-VRB-2s* | 0.05 nM/0.1 |
| LK-VRB-7s* | 0.5 nM/1 |
| LK-VRB-8s* | 0.5 nM/1 |
| LK-VRB-2f | 0.05 nM/0.1 |
| Compare Native VEGF (8-109) | 0.5 nM/1 |

EXAMPLE 11

RIA Assay to Determine Binding of VEGF Variants to KDR and FLT-1 Receptors

An RIA assay was conducted essentially as described in Muller et al., PNAS, 94:7192-7197 (1997) to examine relative binding affinities of several of the VEGF variants (described in Table 2) to the KDR receptor and FLT-1 receptor, as compared to native VEGF 165 or native VEGF (8-109). The results are shown below in Table 6.

Table 6

| Relative Binding Affinity | | |
|---------------------------|--------------|----------------|
| Variant Identifier | KDR Receptor | FLT-1 Receptor |
| Native VEGF 165 | 1 (97 pM) | 1 (37 pM) |
| Native VEGF (8-109) | 12 | 29 |

The figure consists of seven horizontal panels, each representing a time point after the addition of actinomycin D: 0 min, 15 min, 30 min, 45 min, 1 h, 1.5 h, and 2 h. Each panel contains a gel electrophoresis image with multiple lanes. The lanes are labeled at the bottom of each panel with various numbers and letters, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100. The gels show bands representing different proteins, with some bands becoming more prominent over time.

Up-regulation of eNOS by KDR-specific VEGF variants

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In vivo Down-regulation of eNOS by VEGF antagonists

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EXAMPLE 14Modulation of eNOS activity by VEGF

To further elucidate the role of VEGF in modulating the eNOS activity and endogenous NO release, the phosphotyrosine levels of eNOS post-VEGF stimulation were examined. In this time-course study, endothelial cells were first treated with VEGF for 0, 5, 10 15 30 or 60 minutes. Cells were lysed in a buffer containing tyrosine phosphatase inhibitors (sodium vanadate and sodium fluoride). Cell lysates were first immunoprecipitated with an anti-eNOS antibody, and then immunoblotted with an anti-phosphotyrosine antibody. Phosphotyrosine levels were detected by Western blot as described in Example 1. Figure 12 shows that as the VEGF treatment prolongs, the phosphotyrosine levels of eNOS are gradually reduced, which may in turn contribute to eNOS activation and sustained NO release.

Several eNOS associated proteins that attribute to eNOS activity were analyzed for their association/dissociation with eNOS under VEGF treatment.

Endothelial cells were first treated with VEGF for 0 (control), 1, 5, 15, 30 or 60 minutes. Cell lysates were immunoprecipitated with an anti-eNOS antibody, and then immunoblotted with an antibody against α -caveolin, PLC- γ , Hsp90 or Hsp70 for Western blot analysis. The results show that VEGF significantly reduces eNOS association levels of caveolin and PLC- γ , even at short exposure time (1 minute). Meanwhile, VEGF can increase the levels of Hsp90 and Hsp70 associated with eNOS. The results suggested that VEGF can regulate eNOS activity by modulating its association with various proteins.

The foregoing written description is considered to be sufficient to enable one skilled in the art to practice the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

WE CLAIM:

1. A method of treating a nitric oxide (NO) associated disorder in a mammal comprising administering to said mammal an effective amount of vascular endothelial growth factor (VEGF) or VEGF receptor agonist.

5 2. The method of claim 1 wherein said disorder is hypertension, diabetes, angina, thrombosis, heart failure or atherosclerosis.

3. The method of claim 1 wherein said VEGF comprises recombinant human VEGF.

10 4. The method of claim 1 wherein said VEGF receptor agonist comprises a KDR receptor selective variant.

5. The method of claim 1 wherein said VEGF receptor agonist comprises a KDR receptor antibody.

6. The method of claim 5 wherein said KDR receptor antibody comprises a chimeric antibody.

15 7. The method of claim 5 wherein said KDR receptor antibody comprises a human antibody.

8. The method of claim 1 wherein said mammal is a human.

9. The method of claim 1 wherein said VEGF is administered to said mammal by intravenous infusion.

20 10. The method of claim 1 wherein said effective amount of VEGF or VEGF receptor agonist enhances nitric oxide production in said mammal.

11. A method of protecting a mammal from NO associated pathologic conditions, comprising administering to said mammal an effective amount of VEGF or VEGF receptor agonist.

25 12. The method of claim 11 wherein said effective amount of VEGF or VEGF receptor agonist enhances nitric oxide production in said mammal.

13. A method of reducing restenosis in a mammal, comprising administering to mammal undergoing or having undergone angioplasty an effective amount of VEGF or VEGF receptor agonist to up-regulate eNOS
30 expression.

14. A method of stimulating sustained production of endogenous NO in an endothelial cell, comprising exposing the endothelial cell to an effective amount of a VEGF receptor agonist, whereby the endothelial NO synthase (eNOS) in the endothelial cell is up-regulated.

15. The method of claim 14, wherein the VEGF receptor agonist is a VEGF variant having selective binding affinity for KDR receptor.

16. The method of claim 15, wherein the VEGF variant comprises one or more amino acid substitutions at or between positions 17 to 25 of the native VEGF sequence (SEQ ID NO:4).

17. The method of claim 16, wherein the VEGF variant comprises at least the following amino acid substitutions: M18E, Y21L, Q22R and Y25S.

18. The method of claim 15, wherein the VEGF variant comprises one or more amino acid substitutions at or between positions 63 to 66 of the native VEGF sequence.

19. The method of claim 18, wherein the VEGF variant comprises at least the following amino acid substitutions: D63S, G65M, L66R.

20. The method of claim 14, wherein the VEGF receptor agonist is a KDR receptor antibody.

MODULATION OF eNOS ACTIVITY AND THERAPEUTIC USES THEREOF

ABSTRACT OF THE DISCLOSURE

5 The present invention provides uses of VEGF or VEGF receptor agonists for the up-regulation of eNOS expression and activity. VEGF and VEGF receptor agonists are useful in the treatment of or prevention from hypertension, diabetes, angina, thrombosis, atherosclerosis, heart failure, and other conditions or disorders wherein nitric oxide is an important regulator.

1/20

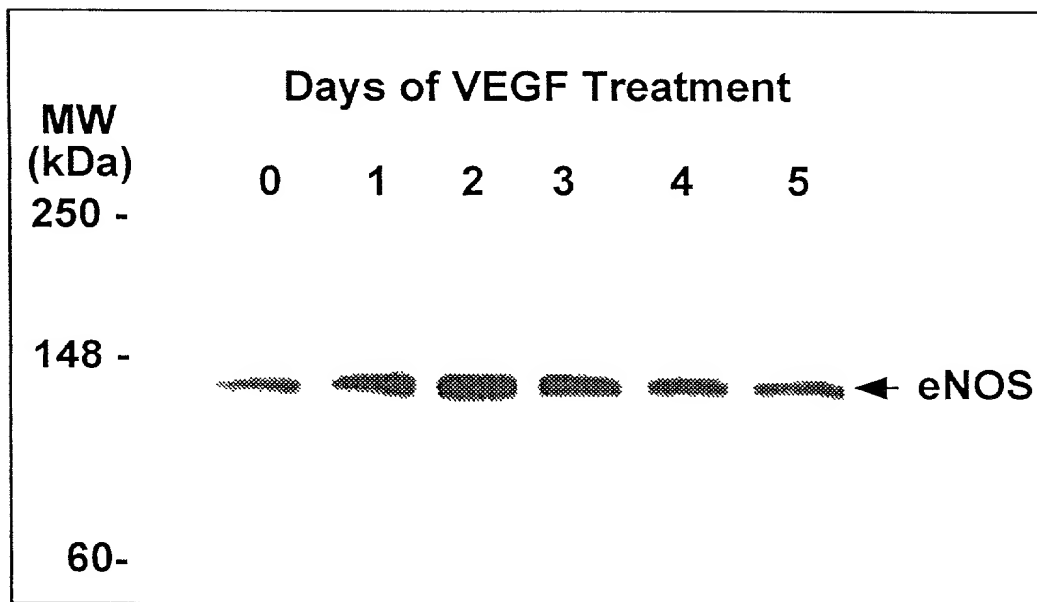


Figure 1A

2/20

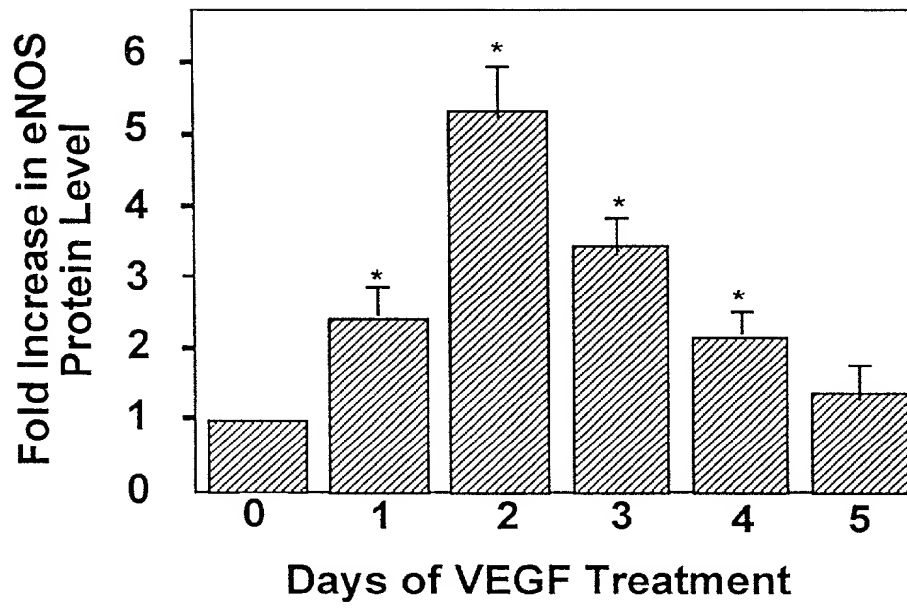


Figure 1B

3/20

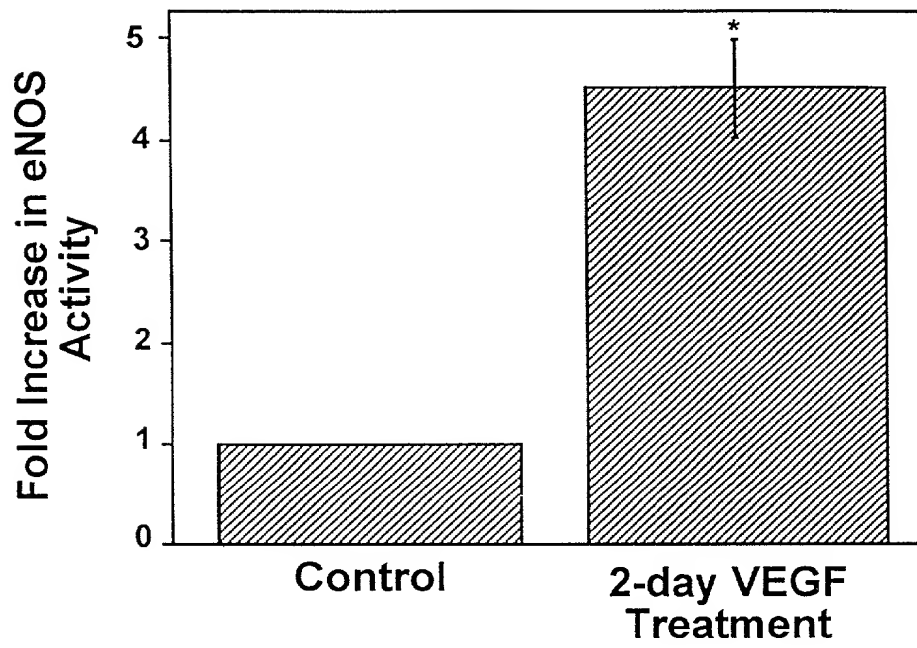


Figure 1C

4/20

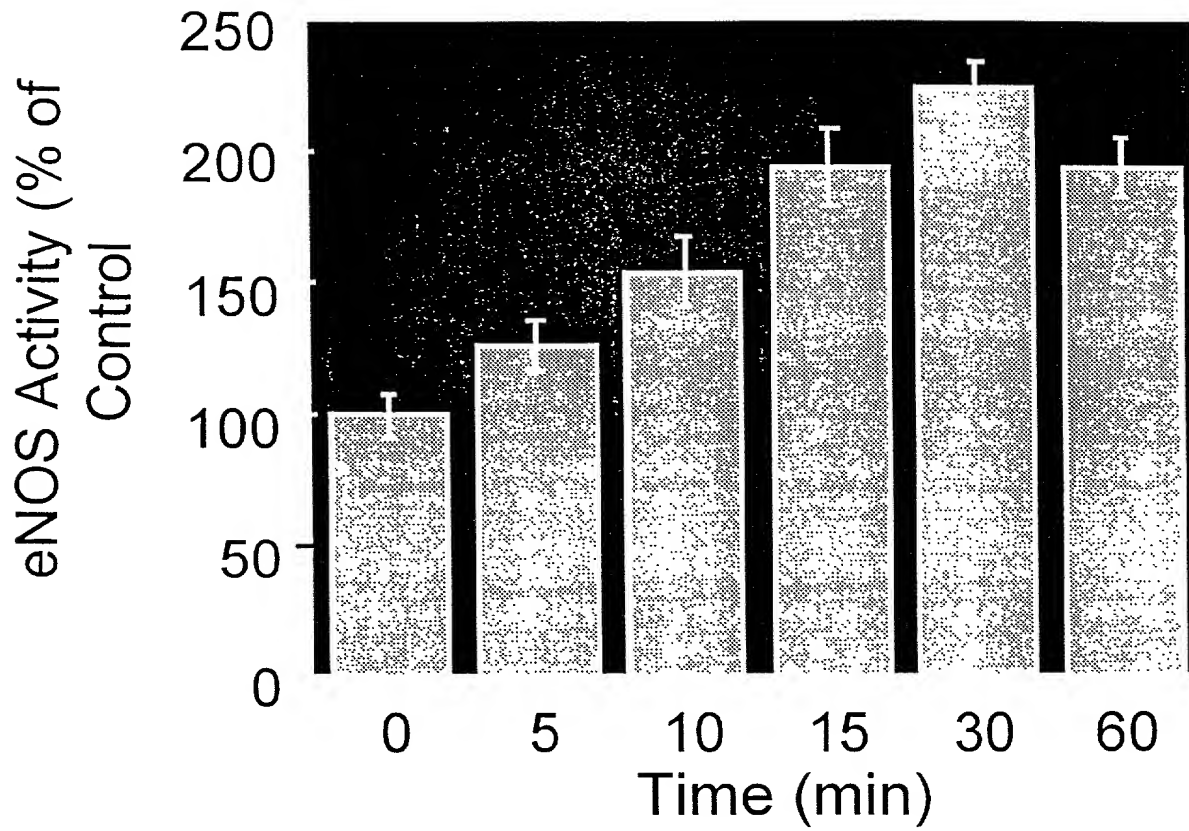
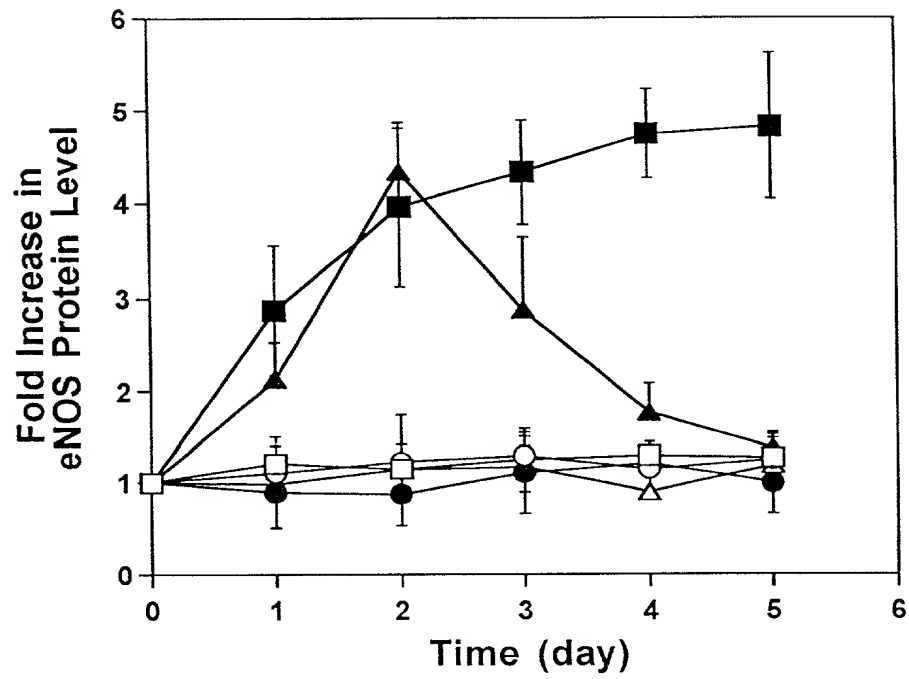


Figure 1D

5/20



- Δ vehicle control
- ▲ VEGF
- L-NAME
- VEGF+ L-NAME
- SNAP
- VEGF+ SNAP

Figure 2



7/20

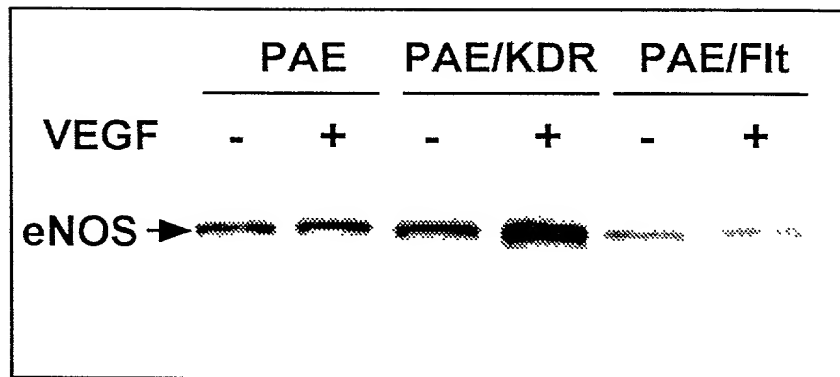


Figure 3B

8/20

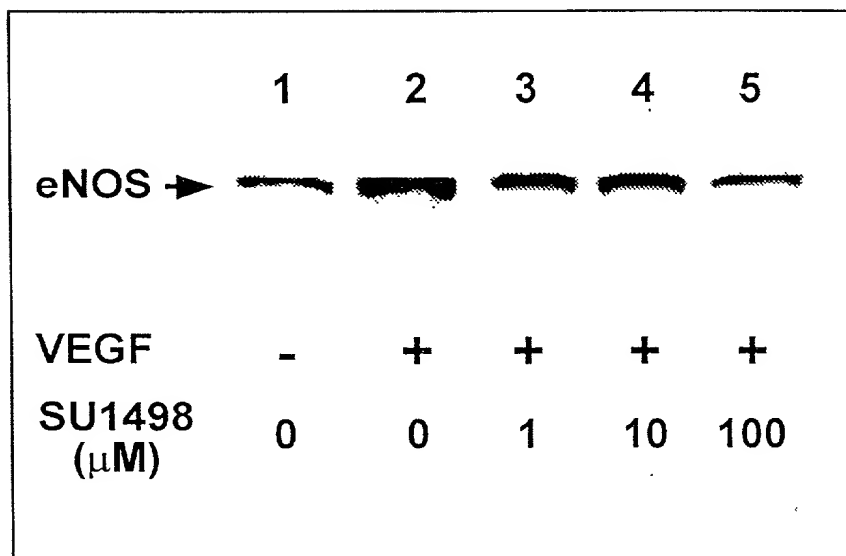


Figure 3C

9/20

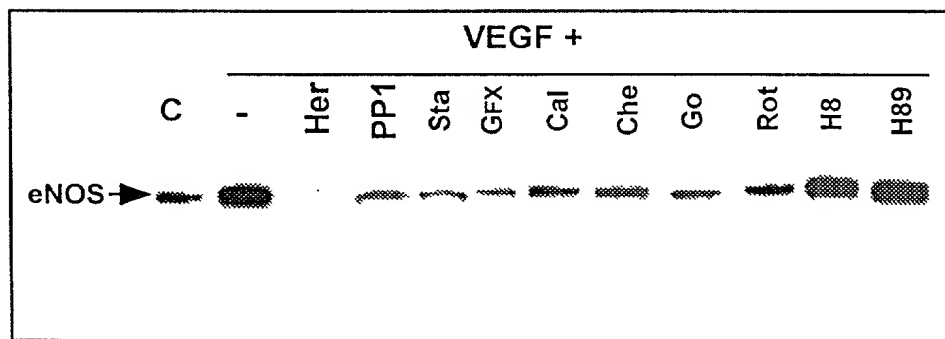


Figure 4A

10/20

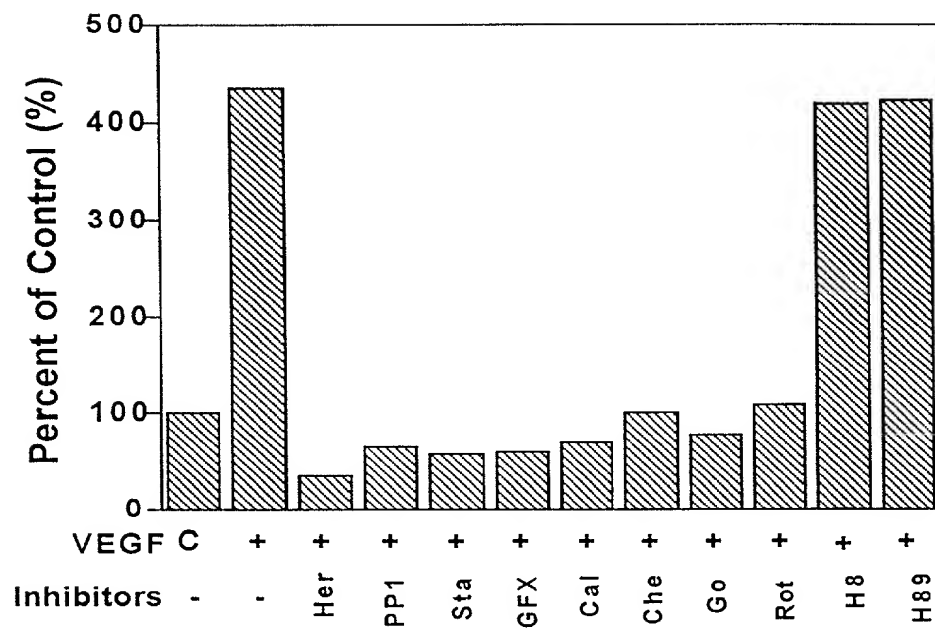


Figure 4B

11/20

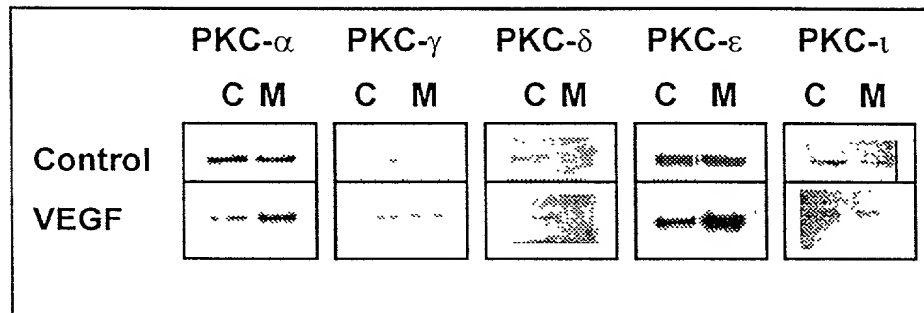


Figure 5A

12/20

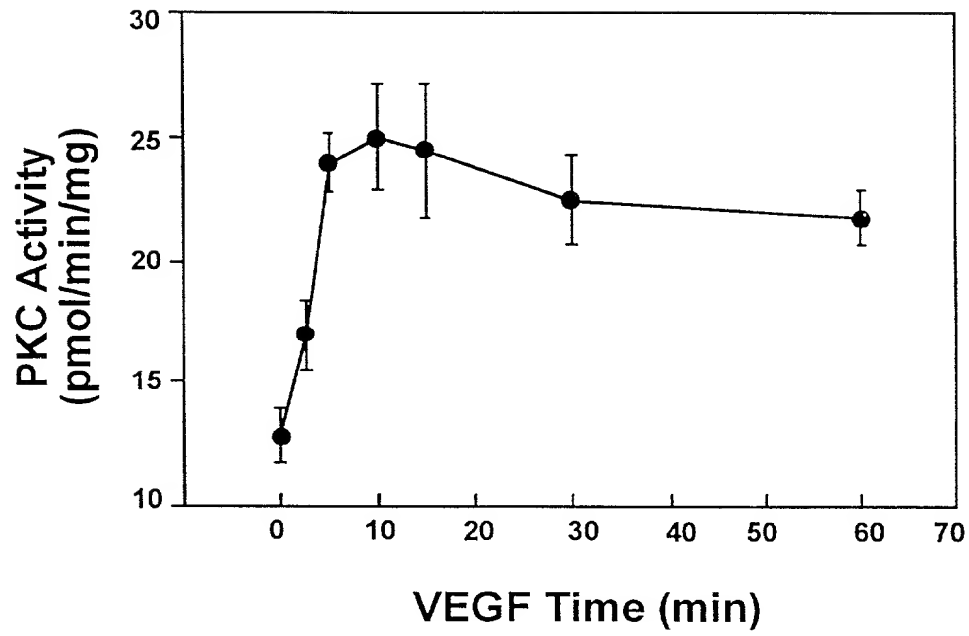


Figure 5B



14/20

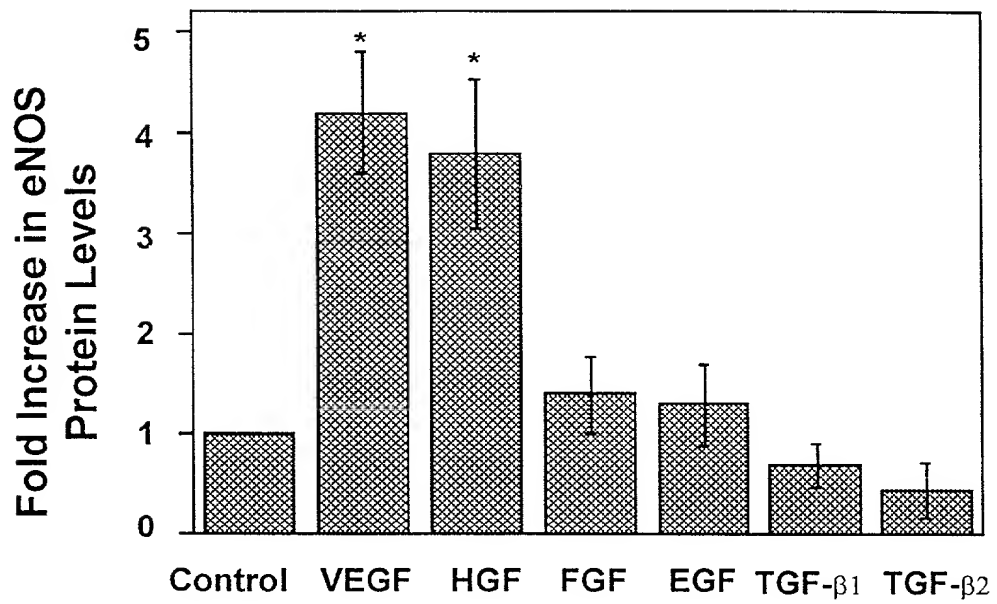


Figure 6

15/20

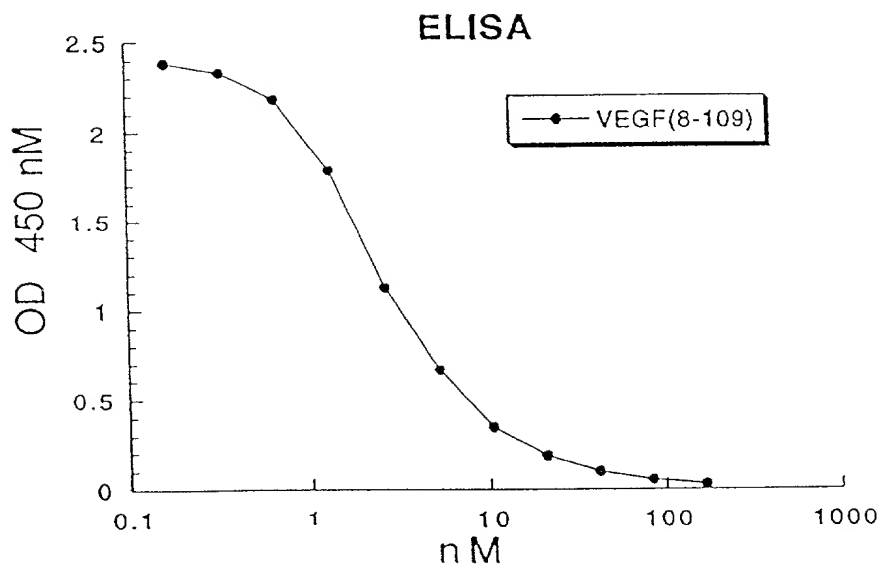


Figure 7

16/20

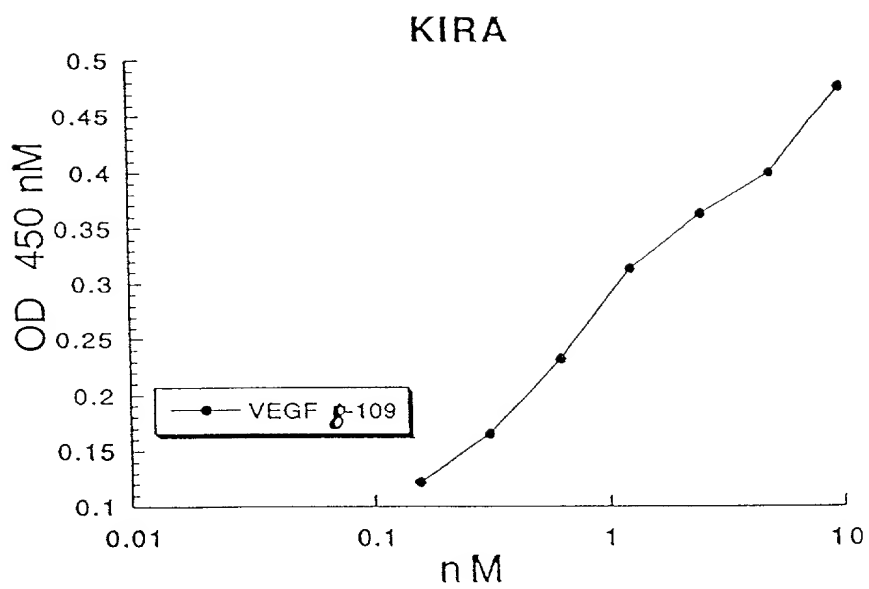


Figure 8

17/20

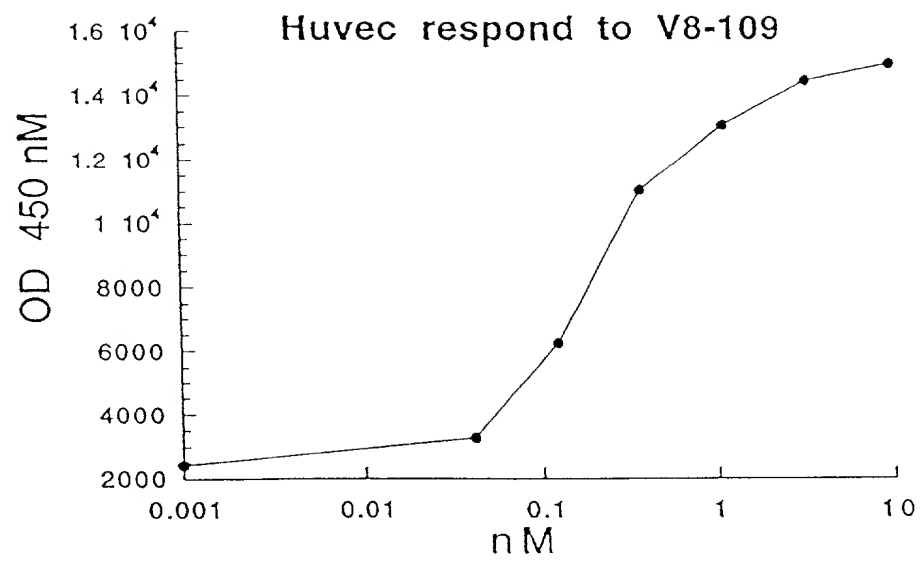


Figure 9

18/20

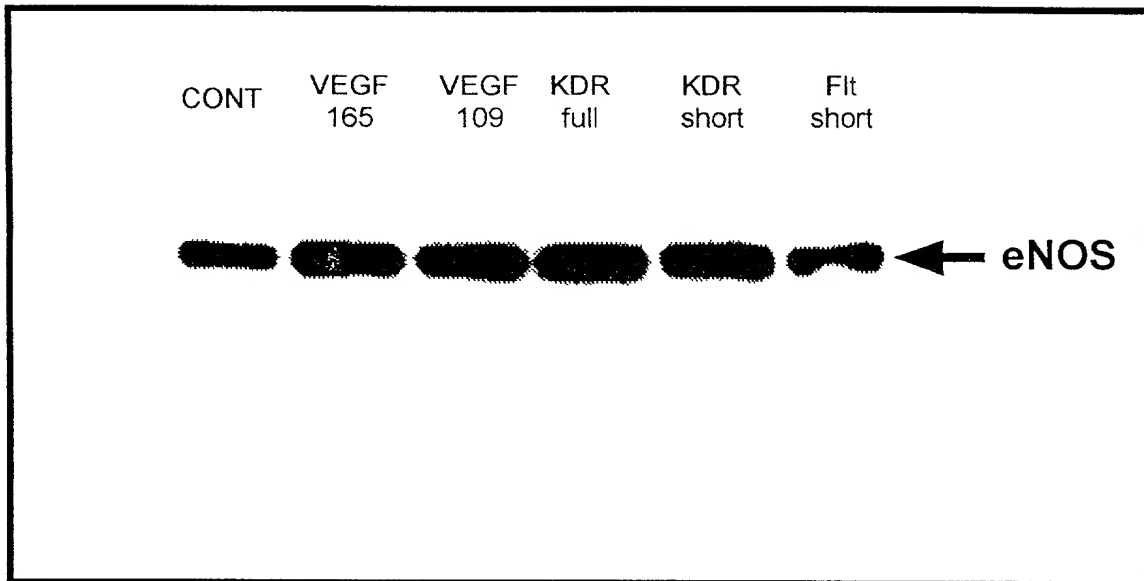


Figure 10

19/20

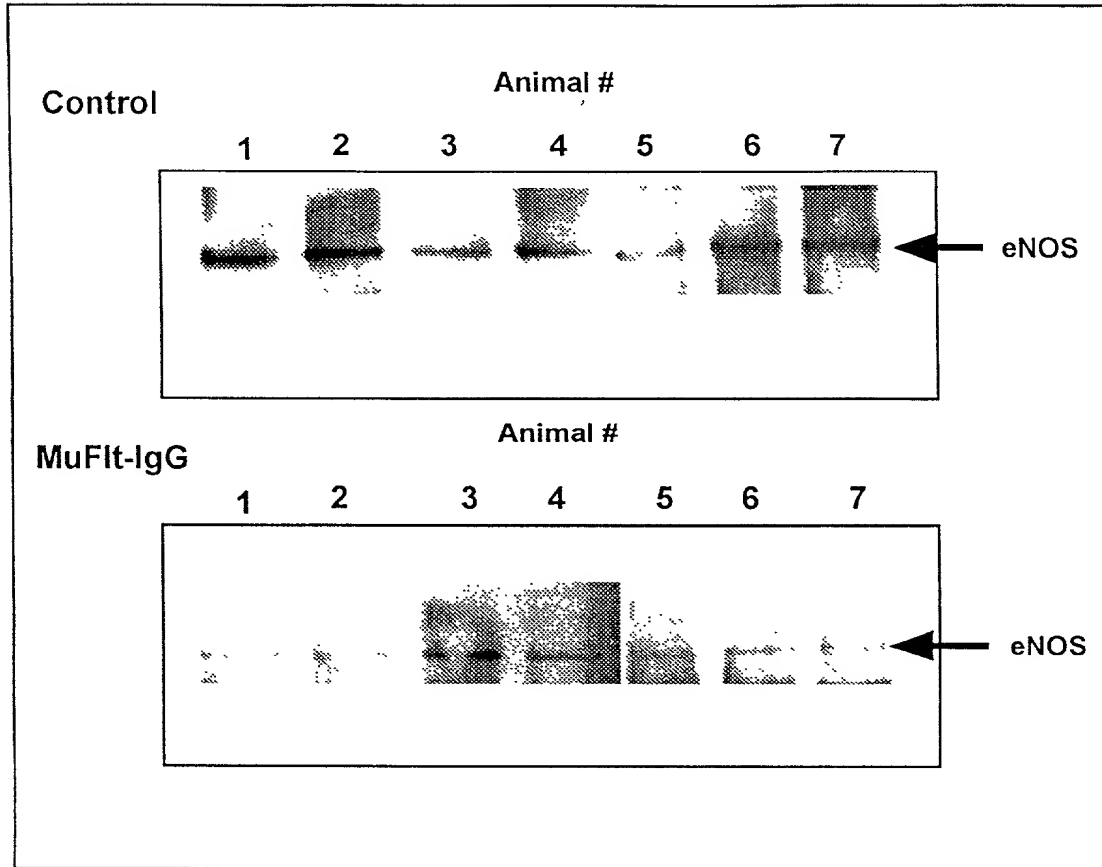


Figure 11

20/20

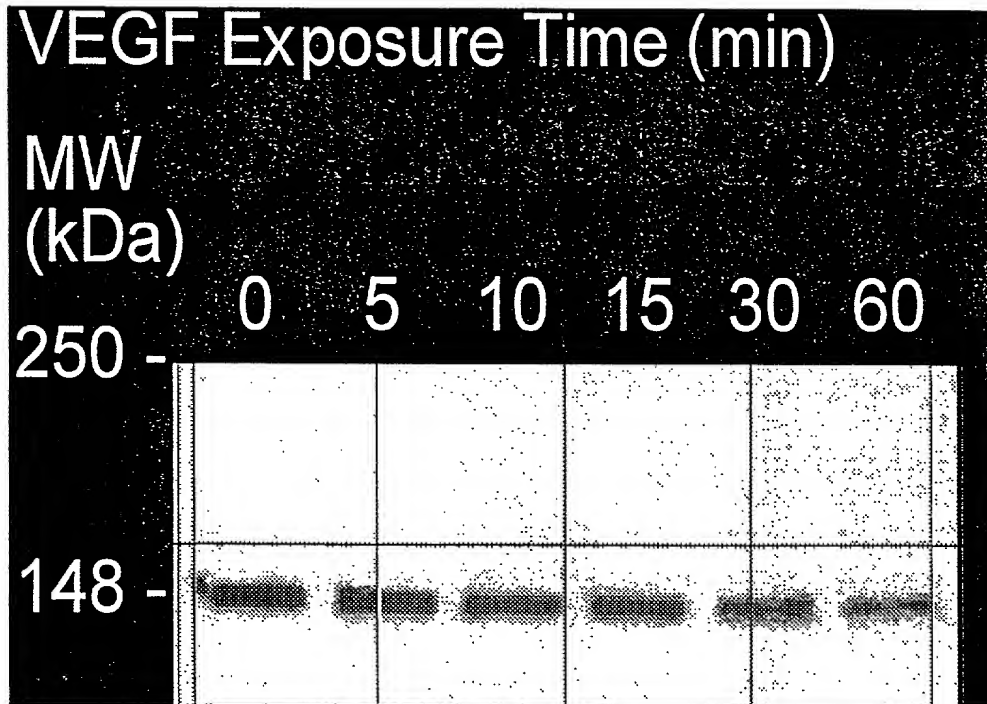


Figure 12

Sequence Listing

<110> Genentech, Inc.
Shen, Ben-Quan
Zioncheck, Thomas

5 <120> MODULATION OF eNOS ACTIVITY AND THERAPEUTIC USES THEREOF

<130> P1735R1PCT

<150> US 60/163,132

<151> 1999-11-02

<160> 4

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<223> Sequence is synthesized.

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<221> unsure

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<223> N at indicated positions may be G, A, T or C; S at indicated positions may be C or G

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aatcgag 57

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00700365-034260

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5

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25

30

35

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 cgggcgggtg acccagcacg gtccctcttg gaattggatt cgccatttta 900
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 <211> 191
 <212> PRT
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 Leu Tyr Leu His His Ala Lys Trp Ser Gln Ala Ala Pro Met Ala
 20 25 30
 Glu Gly Gly Gly Gln Asn His His Glu Val Val Lys Phe Met Asp
 35 40 45
 Val Tyr Gln Arg Ser Tyr Cys His Pro Ile Glu Thr Leu Val Asp
 50 55 60
 Ile Phe Gln Glu Tyr Pro Asp Glu Ile Glu Tyr Ile Phe Lys Pro
 65 70 75
 Ser Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn Asp Glu
 80 85 90
 Gly Leu Glu Cys Val Pro Thr Glu Glu Ser Asn Ile Thr Met Gln
 95 100 105
 Ile Met Arg Ile Lys Pro His Gln Gly Gln His Ile Gly Glu Met
 110 115 120

09-700,806

P1735R1

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| | | | | | | | | | | | | | | | | |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
| | Ser | Phe | Leu | Gln | His | Asn | Lys | Cys | Glu | Cys | Arg | Pro | Lys | Lys | Asp | |
| | | | | | 125 | | | | | 130 | | | | | 135 | |
| | Arg | Ala | Arg | Gln | Glu | Asn | Pro | Cys | Gly | Pro | Cys | Ser | Glu | Arg | Arg | |
| | | | | | 140 | | | | | 145 | | | | | 150 | |
| 5 | Lys | His | Leu | Phe | Val | Gln | Asp | Pro | Gln | Thr | Cys | Lys | Cys | Ser | Cys | |
| | | | | | 155 | | | | | 160 | | | | | 165 | |
| | Lys | Asn | Thr | Asp | Ser | Arg | Cys | Lys | Ala | Arg | Gln | Leu | Glu | Leu | Asn | |
| | | | | | 170 | | | | | 175 | | | | | 180 | |
| 10 | Glu | Arg | Thr | Cys | Arg | Cys | Asp | Lys | Pro | Arg | Arg | | | | | |
| | | | | | 185 | | | | | 190 | | | | | | |

09-700,806

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office and citizenship are as stated below next to my name,

I believe I am the original and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

MODULATION OF eNOS ACTIVITY AND THERAPEUTIC USES THEREOF

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as United States application Serial No. _____ on _____ and was amended on _____ (if applicable.).

☒ was filed as PCT international application Number PCT/US00/30294 on 02 November 2000 and was amended under PCT Article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate or of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, any foreign application(s) for patent or inventor's certificate or, of any PCT international application having a filing date before that of the application(s) on which priority is claimed.

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119(a)-(d):

| COUNTRY | APPLICATION NUMBER | DATE OF FILING (day, month, year) | PRIORITY CLAIMED UNDER 35 USC 119 |
|---------|--------------------|--------------------------------------|--------------------------------------|
|---------|--------------------|--------------------------------------|--------------------------------------|

I hereby claim benefit under 35 U.S.C. 119(e) of any United States Provisional Application(s) listed below.

| Application No.(s) | Filing Date |
|--------------------|------------------|
| 60/153,132 | 02 November 1999 |

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s) of any PCT international application(s) designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application(s) and the national or PCT international filing date of this application:

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Continued)

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

| U.S. APPLICATIONS | | STATUS (check one) | | |
|-------------------------|------------------|--------------------|---------|-----------|
| U.S. Application Number | U.S. Filing Date | Patented | Pending | Abandoned |
| | | | | |
| | | | | |
| | | | | |

PCT APPLICATIONS DESIGNATING THE U.S.

| PCT Application No. | PCT Filing Date | U.S. Serial Numbers | | | |
|---------------------|-----------------|---------------------|--|--|--|
| | | | | | |
| | | | | | |

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.



09157

PATENT TRADEMARK OFFICE

Send correspondence to Genentech, Inc.

Attn: Steven X. Cui

Telephone: 650-225-8674

I hereby declare that all statements made herein of my own knowledge and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issue thereon.

The undersigned hereby authorizes the U.S. attorney or agent named herein at accept and follow instructions from his foreign patent agent as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

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